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PARENTERAL NUTRITION EFFECTS ON DRUG METABOLISM IN PROTEIN-CALORIE MALNOURISHED RATS

by

Zhongping Mao

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **PARENTERAL NUTRITION EFFECT ON DRUG METABOLISM IN PROTEIN CALORIE MALNOURISHED RATS** submitted by Zhongping Mao in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in Pharmacy and Pharmaceutical Sciences.

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DEDICATION

This work is dedicated to my family and friends, whose love and support made this thesis possible.

ABSTRACT

The effects of parenteral nutrition (PN) on drug metabolism in protein-calorie malnourished (PCM) rats were investigated using *in vitro* and *in vivo* methods. The principal studies that were conducted in this project included: (1) An evaluation of protein-calorie malnourishment effects on *in vitro* drug metabolism in pediatric rats using testosterone as a probe; (2) An evaluation of protein-calorie malnourishment effects on *in vitro* drug metabolism in adult rats; (3) An investigation of PN effects on *in vitro* drug metabolism in malnourished adult rats; (4) An evaluation of protein-calorie malnourishment effects on nifedipine pharmacokinetics in malnourished adult rats; (5) An evaluation of PN effects on nifedipine pharmacokinetics in malnourished adult rats; (6) An investigation of the mechanism which caused the alteration of drug metabolism in malnourished rats.

To quantify testosterone metabolites in rat liver microsomes, a simple and sensitive HPLC assay method was developed and validated for subsequent *in vitro* studies.

In vitro results showed that protein-calorie malnourishment diminished microsomal protein and total P450 contents in livers of both pediatric and adult rats. Capacity and affinity of CYP2C11, CYP3A, and CYP2A1 in PCM both pediatric and adult rats were also impaired. PN diminished the microsomal protein and total P450, and impaired the capacity and affinity of CYP2C11 and CYP3A in healthy rats. PN further impaired the capacity and affinity of CYP2C11 and CYP3A in PCM rats.

In vivo nifedipine clearance was significantly impaired and $t_{1/2}$ values increased in PCM adult rats and PN-treated healthy adult rats. Volume of distribution at steady state was significantly lower in PCM rats. PN further impaired nifedipine clearance in PCM adult rats. Volume of distribution did not show a significant difference between enteral nutrition and PN groups.

Rat growth hormone (rGH) profiles did not differ significantly between PCM rats and healthy rats. It is unlikely that an alteration of rGH production caused the alteration of drug metabolism in PCM rats.

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GLOSSARY OF ABBREVIATIONS AND SYMBOLS

Variable	Description
λ	lambda-elimination rate constant
α	probability of making a type I error
µL	microliter(s)
µM	micromolar
AAG	α_1-acid glycoprotein
ANOVA	analysis of variance
$AUC_{0\text{-LAST}}$	area under the plasma concentration vs. time curve from time zero to last sample time
$AUC_{0\text{-}\infty}$	area under the plasma concentration vs. time curve from time zero to infinity
UDPGA	Uridine 5'-diphospho-α-D-glucuronic acid
GSH	glutathione
C	drug concentration in plasma
HIV	human immunodeficiency virus
CL_{TB}	total body clearance
$C_{T(LAST)}$	drug concentration at the last sampling time point
CYP	cytochrome P450
DHEA	dehydroepiandrosterone
DHEAS	dehydroepiandrosterone sulfate
EDTA	ethylenediaminotetraacetate
g	gram(s)
GH	growth hormone
HCl	hydrochloride
HPLC	high-performance liquid chromatography
hr	hour(s)
H_X	hypophysectomy
I.D.	internal diameter
I.S.	internal standard
iv	intravenous
K	the estimated elimination rate constant
kg	kilogram(s)
K_m	Michaelis constant
L	liter(s)
M	molar
mg	milligram(s)
min	minute(s)
mL	milliliter
mm	millimeter(s)
mmol	millimole(s)
MTBE	methyl-t-butylether
MVI	multivitamin

n	number of observations
NADPH	nicotinamide adenosine dinucleotide phosphate, reduced
NaOH	sodium hydroxide
NM	nanometers
°C	degree celsius
OD	outside diameter
P	probability value of a test result, given that the null hypothesis is true
PEG	polyethylene glycol
pH	negative logarithmn (base 10) of the hydrogen ion concentration
PK	pharmacokinetics
PN	parenteral nutrition
QC	quality control
Q_H	total hepatic blood flow
rGH	rat growth hormone
S	substrate concentration
SD	standard deviation
SD	Sprague-Dawley
t	time
$t_{1/2}$	elimination half-life
t_{last}	last quantifiable sampling time
UV	ultra-violet
V/V	volume per volume
V_{MAX}	Maximum velocity
V_{ss}	volume of distribution at steady state
WHO	World Health Organization
PCM	protein-calorie malnourished
NIDDK	National Institute of Diabetes and Digestive and Kidney Diseases

CHAPTER 1

INTRODUCTION

1.1 NUTRITION STATUS IN THE WORLD AND THE NATURE OF THE PROBLEM

The major nutritional disorder, which occurs globally and accounts for severe morbidity and mortality, is protein-calorie malnutrition. According to The World Health Report 1999 [1], the malnutrition population in the world stood at around 30-35% in some African countries, 5-35% in south and north American continents, and 18-43% in some sections of Asia. About 1% of people in the World Health Organization (WHO) region died of nutritional deficiencies, of which more than half was caused directly by protein-calorie malnutrition. This is just for the direct cause of death; actually malnutrition is estimated to be the single most important risk factor for disease, being responsible for 16% of the global burden in 1995. Malnutrition, in the form of protein-calorie inadequacy or micronutrient malnutrition, primarily of iron, vitamin A and iodine, often contributes to premature death, poor health, blindness, growth stunting, mental retardation, learning disabilities and low work capacity.

The inception, progression and ultimate manifestations of malnutrition are related to dietary intake of proteins and calories and are further compounded by an impoverished environment, repeated infections or infestations and inadequate management. Malnutrition has its inception in the womb; a relatively high proportion of infants starts

with low birthweight, and when they are weaned, duration and degree of inadequate dietary intake determine the dimension of nutritional problems.

Protein-calorie malnutrition covers a wide spectrum of pathological conditions [2,3]. The manifestations vary widely according to age, duration of food deprivation and associated conditions. The manifestations and consequences of protein-calorie malnutrition are diverse and alarming. Severe forms of this disease are kwashiorkor and marasmus. Although the terms kwashiorkor and marasmus were introduced long ago, there is no general definition. Marasmus is characterized by loss of weight, emaciation, loss of turgor in the skin, almost total disappearance of subcutaneous fat and atrophy of the muscles. Plasma albumin concentrations may or may not be low. Patients with kwashiorkor manifest inadequate growth for children, loss of muscular tissue, oedema and hepatomegaly. Apathy, discolored sparse hair and mental changes are common features. Patients with severe protein-calorie malnutrition show features of both clinical entities of kwashiorkor and marasmus.

Malnutrition, which is assumed to be primarily concentrated in the world's poorest and most disadvantaged populations, is also a big concern of developed countries since it occurs not only because of lack of food, but also in maldigestion, malabsorption and chronic hemodialysis especially among hospitalized population and elderly people. Patients with absorption disorders, renal failure, human immunodeficiency virus (HIV), cancer, hepatic diseases and other diseases are always malnourished. It was reported that more than 50% of hospitalized patients are malnourished [4].

Medicines are prescribed for patients, so the metabolism of the patient should influence the dosage design. If the rate of metabolism is different between malnourished subjects and subjects with normal nutritional status, then clinicians might want to take the patient's nutrition status into consideration and redesign a dosage regimen to maximize therapeutic benefits.

1.2 MALNUTRITION AND PHARMACOKINETICS

Malnutrition is a global problem and a variety of nutritional disorders are encountered within a given population. As a consequence of numerous pathological conditions, which are encountered in malnutrition, there may be a wide range of changes in pharmacokinetic or pharmacodynamic processes of xenobiotics.

Drugs are used as widely in malnourished as in well-nourished populations. In fact, certain therapeutic classes of drugs such as anti-inflammatory, antifungal, antivirus, and anti-anaemia agents are used more often in the malnourished. Rational use of drugs is based on sound pharmacokinetic and pharmacodynamic principles, and knowledge of these and the factors modifying the fate of the drug in the body are essential for the proper management of patients. It is now recognized that diet and nutritional status are two important variables that can alter drug metabolism and disposition [5-7].

1.2.1 Malnutrition and Drug Absorption

Drugs are most frequently administered by the oral route. Absorption has to be rapid and sufficiently extensive for the achievement of therapeutic plasma concentrations. When a drug is administered orally, it undergoes various processes before entering the systemic circulation and reaching its site of action. Important physical and physiological factors which determine drug absorption include the pH of the intestine and the intestinal transit time, surface area of the gastrointestinal tract, intestinal blood flow, gut bacteria and gut wall metabolism of drugs [8]. In addition, a drug has to pass through the intestinal mucosa and liver before entering the systemic circulation, and therefore metabolic adaptations can also ultimately determine the amount of drug in the systemic circulation. The 'first-pass' effect, or presystemic elimination, is an important determinant of drug availability. As a result, absorption will depend on the drug's solubility in water and fat, its ionization at various pH values, and its availability at the site of absorption in the gastrointestinal tract [9].

Nutritional deficiencies can alter gastrointestinal structure and function and, as a result, drug absorption. Mucosal atrophy of the bowel and rapid intestinal transit (diarrhoea) may occur in PCM subjects. Many reports document that severe malnutrition impairs absorption. The rate of absorption of chloramphenicol is diminished and the time to attain peak blood level is delayed in severe PCM patients [10]. The absorption of chloramphenicol is erratic in Ethiopian children with kwashiorkor [11]; plasma concentrations after oral administration of chloramphenicol palmitate indicate a decrease

in absorption. Absorption of nutrients given as medicaments, such as iron is decreased in severe malnutrition [12]. Peak plasma concentrations of chloroquine and the mean area under the plasma concentration-time curve are lower in children with kwashiorkor than in healthy children when chloroquine is given orally. Impaired absorption has been offered as an explanation for the lower plasma concentrations for some severe malnourished subjects [13]. Absorption of drugs such as tetracycline, rifampicin and anticonvulsants are also documented to be low in adult malnutrition [14,15].

The impairment in the absorption of drugs generally happens in severely malnourished subjects. Most severely malnourished patients have gross oedema. As many hospitalized patients with protein-calorie malnutrition receive antibiotics by the intramuscular route (i.m.), the bioavailability of those drugs could be impaired. However, according to reports by Buchanan et al. [16-18], the bioavailability values of penicillin, tobramycin and cefoxitin were acceptable except in the presence of absolutely gross oedema (greater than 30% of body weight). It was concluded that, except in the grossly oedematous or shocked patients, intramuscular antibiotic administration was therapeutically acceptable. So far the data at hand do not suggest that the patient with mild to moderate protein-calorie malnutrition, who might be treated with oral medication, has a significant absorptive problem [19].

1.2.2 Malnutrition and Drug Distribution

1.2.2.1 Volume of Distribution

Drug distribution to tissue is usually assessed by apparent volume of distribution. Shastri and Krishnaswamy [20] documented that the volume of distribution of tetracycline was reduced in acute PCM. Buchanan's group reported similar findings with gentamicin [21], cefoxitin [22], isoniazid [23], acetanilide [24] and antipyrine [25]. In adult malnutrition, tissue uptake and binding is also observed to be low [20,26]. This would imply higher serum drug concentration for the same mg/kg dose than in well-nourished individuals if other pharmacokinetic parameters remain the same. Nevertheless, the change of the volume of distribution of most of the drugs was not significant [17].

1.2.2.2 Protein Binding

Malnutrition could alter plasma protein profile and the rate of tissue protein synthesis and turnover [20,26,27]. Drug-protein binding therefore may be expected to be altered by various degrees of malnutrition. Nutrient-related substances such as fatty acids, bilirubin, hormones and thyroxine, which also bind to albumin, can modify drug-protein binding by displacing drugs from binding sites [20,28,29]. It was reported that salicylate, oxacillin, and dicloxacillin showed significant displacement of bilirubin from albumin and the binding of salicylate, sulfadiazine and thiopental with albumin was reduced with high concentrations of free fatty acids [30].

One of the hallmarks of kwashiorkor is hypoalbuminaemia [31]. Shastri and Krishnaswamy have reported diminished tetracycline binding to plasma proteins [20]. Krishnaswamy's group also made similar observations with phenylbutazone [32]. Buchanan [17] studied *in vitro* binding of 18 drugs in serum of children with kwashiorkor using equilibrium dialysis. Drugs studied included chloramphenicol, penicillin, sulphamethoxazole, gentamicin, cloxacillin, flucloxacillin, streptomycin, ethambutol, ethionamide, isoniazid, para-aminosalicylic acid, rifampicin, phenobarbitone, phenytoin, thiopentone, digoxin, chloroquine and dicoumarol. At all concentrations studied, drug binding to kwashiorkor serum was found to be much less than with the control serum. Studies in adults on drugs such as phenylbutazone, doxycycline, tetracycline, sulphadiazine and rifampicin indicated the same result.

Total clearance of drug from the body is affected by the extent to which drugs are bound to plasma proteins [33]. Total clearance is directly proportional to the concentration of unbound drug available for low extraction ratio drugs [34]. Total clearance of the drug from the body is believed to be a function of plasma protein binding and drug half-life is a function of tissue binding for drugs with a high volume of distribution. If volume of distribution of a drug is less than 100 ml/kg, the volume of distribution is highly dependent on plasma protein binding [35]. With linear kinetics, drug half-life is inversely proportional to the free fraction of the drug in tissues. A reduction in the extent to which a drug is protein-bound may result in an increased rate of drug delivery to metabolic and receptor sites, the uptake and metabolism being proportional to

the concentration of unbound drug in plasma. However, increased free drug concentrations and the capacity of the liver and kidney to eliminate drugs seem to vary in patients with severe PCM. Nevertheless, toxicities are likely to be enhanced for highly protein-bound drugs, which have narrow therapeutic windows.

1.2.3 Malnutrition and Drug Metabolism

1.2.3.1 Oxidative Metabolism in Malnutrition

Enzymes that metabolize drugs are located in endoplasmic reticula of liver, intestinal mucosa, skin, lungs, and other organs such as brain, kidneys, placenta. Metabolism is one of the major determinants of drug clearance and accordingly, of drug concentration and action. Consequently drug metabolism has a direct effect on the pharmacological and toxicological activities of drugs. Most nutrients participate directly or indirectly in the functioning of enzyme systems involved in the biotransformation of drugs, such as competing with enzymes for metabolic and protein binding sites, and inducing or inhibiting CYP [36]. For instance cruciferous vegetables induce CYP1A2 and grapefruit juice inhibits CYP3A4. Since the essential amino acids are bricks for protein synthesis, nutrition is critical for enzyme synthesis.

There are some reports in the literature that document a malnourishment effect on drug metabolism in animals. Anthony [37] used 90-110 g rats for a protein deficiency study on drug metabolism in rat liver microsomes. They found microsomes from rats fed

a low protein (0.5% or 1%) diet had a rate of ethylmorphine N-demethylation equivalent to 16% of the control rate.

The study carried out by Leakey's group [38] on effects of aging and caloric restriction on hepatic drug metabolizing enzymes in rats showed that caloric restriction decreased the age-related changes in hepatic testosterone metabolism. They also found increased hepatic microsomal testosterone 6β-hydroxylase activity in the experimental group when malnourished rats were fed with 60% of the control ration diet.

Catz [39] found that in neonatal animals with malnutrition, the difference in the rate of metabolism was not uniform. For oxidative pathways, an increase was found using aminopyrine and benzpyrene as substrates. However, the rate of aniline metabolism was not altered. For reductive pathways, a decrease was observed with 2 substrates: p-nitrobenzoic acid and neoprontosil.

Effects of malnutrition on animal drug metabolism showed a complex picture, and results from human studies are also controversial.

It was reported that malnutrition enhanced drug metabolism in humans. In a single oral dose study in adult male subjects with different nutritional status, it was found that the elimination half-life of phenylbutazone was significantly shorter and plasma clearance accelerated in undernourished patients who had significant weight deficit and low serum albumin concentration [32]. Doxycycline clearance was also reported to be higher in malnourished subjects [40]. These researchers postulated that the increase in clearance is

probably due to lower plasma protein binding of the drug and/or induction of drug metabolism.

Several other investigators studied the effect of malnutrition on hepatic drug-metabolizing enzymes, as indicated by antipyrine clearance. These studies were mostly performed in children with severe manifestations of protein-calorie malnutrition. Buchanan studied antipyrine pharmacokinetic in kwashiorkor children [41] and found that antipyrine half-life was longer than that observed in healthy children. Narang et al. [42] and Homeida et al. [43] performed similar studies. They also found the antipyrine half-life to be longer than that reported for healthy individuals. In contrast, theophylline metabolism was reportedly not affected by malnutrition [44]. The data indicated that none of the parameters such as half-life or clearance was significantly different from those in healthy control subjects.

It is clear that both animal and human studies provided divergent results for phase I metabolism in PCM subjects. It is the objective of this study to examine what is the effect of malnutrition on total P450 and specific isoenzymes in rats and find out the real effect of malnutrition on drug metabolism.

1.2.3.2 Conjugation in Malnutrition

Rates of conjugation are as much dependent upon the *in vivo* availability of the cofactors such as Uridine 5'-diphospho-α-D-glucuronic acid (UDPGA) and glutathione (GSH) as they are on the enzyme levels. Decreases in conjugation can be due to limited

supply of cofactors [45]. The levels of these cofactors are subject to nutritional stresses. UDPGA and GSH levels are known to be lower during starvation and protein deficiency [46,47]. So it is expected that conjugation would be impaired.

Mehta and colleagues [48,49] studied chloramphenicol pharmacokinetics in malnourished children. When the drug dose was 50 mg/kg, only 35 to 55% of the drug was excreted in conjugated form in malnourished subjects, compared to 75 to 80% in control subjects after oral administration. Elimination of chloramphenicol was delayed, with a concomitant increase in the area under the plasma concentration vs. time curve (AUC) in the malnourished. Steady-state concentrations of chloramphenicol were higher in severe than in mild PCM.

Paracetamol (acetaminophen), a commonly used drug, is eliminated mainly by conjugation. The major routes of conjugation are through glucuronidation and sulphation [50]. When paracetamol was administered to a group of patients with protein-calorie malnutrition, urinary recovery of glucuronide and sulphate metabolites was much less than in healthy subjects [51]. The AUC in PCM subjects was more than three fold higher than in healthy controls.

Studies in adults on the acetylation profile of sulphadimidine did not show any differences between well-nourished and undernourished adults [52]. The glucuronide and glutathione conjugation reactions which inactivate the highly reactive electrophilic intermediates of benzopyrene [53,54] and aflatoxin [55] produced by the mixed function

oxidase, however, showed no changes. The influence of malnutrition on conjugation may be determined by the degree of malnutrition and the type of conjugation.

1.2.3.3 Malnutrition and Drug Excretion

The kidney appears to be susceptible to changes in dietary intake of protein and calories in PCM subjects. Reduction in glomerular filtration and renal plasma flow was documented in malnourished children [56]. The protein intake is positively correlated with glomerular filtration and endogenous creatinine clearance. Studies by Bosch et al. [57] suggested that renal functional reserve is related to protein intake.

Buchanan et al. studied four antimicrobials, gentamicin [21], cefoxitin [22], penicillin [58], tobramycin [59] in PCM children, all of which were insignificantly bound to plasma proteins and were renally excreted. They found that with the exception of cefoxitin, $t_{1/2}$ values were significantly prolonged at the time of admission to hospital compared to after recovery with nutritional therapy. With all the drugs, clearance increased with rehabilitation. Since these antibiotics are excreted by renal tubules, the change in pharmacokinetics is probably a reflection of a reduction of tubular function.

Shastry and Krishnaswamy [20] examined the effect of malnutrition on the elimination of tetracycline, a drug that does not undergo any metabolism and is excreted unchanged, in 9 well-nourished and 10 undernourished adults. They showed that when tetracycline was given intravenously, $t_{1/2}$ was 7.24 ± 0.6 h in the well-nourished group and 4.1 ± 0.28 h in the undernourished group. The mode of administration of the drug or the

dose administered did not affect half-life. In the undernourished subjects, the protein binding of the drug and the relative volume distribution were also significantly lower. The elimination rate of the drug, however, was significantly higher compared to the well-nourished subjects. This fall in $t_{1/2}$ could be associated with a concomitant increase in elimination rate; it also could be related to alterations in plasma protein binding.

A study conducted by Bravo et al. [60] using gentamicin as a probe drug in malnourished children did not show a significant difference in either clearance or $t_{1/2}$, except that volume of distribution was slightly increased in the malnourished group.

The discrepancy in the results of the above reports in the literature could be due to the model drug used or a difference in the type of malnutrition. Different drugs may have differences in protein binding, volume of distribution and elimination. Malnutrition may influence these parameters differently. For tetracycline, in the malnourished subjects, protein binding of the drug was reduced; therefore, it could cause an increase in renal clearance. On the other hand, with renally cleared drugs like penicillin, gentamicin, tobramycin and cefoxitin which are insignificantly bound to plasma proteins, the changes in clearance values were mainly determined by alteration in renal function. Different types of malnutrition may influence renal clearance differently. Acute and severe malnutrition may cause renal pathophysiological alterations more, while chronic or mild malnutrition may not affect renal clearance to any great extent [56].

1.3 NUTRIENT COMPONENTS AND DRUG DISPOSITION

Studies to identify dietary components that can influence drug metabolism have been conducted. The metabolic clearance of antipyrine was increased in patients following consumption of a high protein diet for two weeks [61]. The consumption of a high carbohydrate diet for the same period of time did not significantly alter antipyrine clearance from its baseline values determined when patients received their usual diet. Fagan et al. [62] have also observed in human volunteers that a high-protein diet (40% of total calories) caused a higher metabolic clearance of theophylline and propranolol than a low protein diet (10% of total calories). These results indicate that dietary protein can accelerate the metabolism of these representative substrates of the mixed function oxidase system in human.

1.4 RATIONALE FOR CHOOSING RAT AS AN ANIMAL MODEL

The rat is the most widely used experimental animal in the field of clinical nutrition and drug metabolism, which provides a large database for comparison. Rat CYP2C11 accounts for about one third of the total CYP; there are more than two hundred other isoenzymes in Sprague-Dawley (SD) male rat liver [63]. CYP2C11 metabolizes a host of xenobiotics such as benzphetamine, aminopyrine, ethylmorphine, benzopyrene and warfarin [64]. It is responsible for stereospecific metabolism of endogenous steroids. For example, 2α- and 16α- hydroxylation of testosterone and 16α-hydroxylation of estradiol are catalyzed by CYP2C11 [65]. Most importantly, CYP2C11 in rat shares most of the

substrates with human CYP3A4, which is a major isoenzyme in human [66]. Moreover the small size and low cost of rats and their availability, and ease of handling and maintenance make them attractive for laboratory use. Therefore SD male rats were chosen for evaluating the effects of malnutrition and PN on drug metabolism.

1.5 RATIONALE FOR CHOOSING TESTOSTERONE AS A MODEL DRUG FOR *IN VITRO* STUDY

CYP3A4 is the most abundant isoenzyme (up to 28% of the total) in human liver. It is reported that human CYP3A4 is involved in about 60% of oxidations of clinical drugs [67]. One would assume that the elimination rate of a large number of drugs will be altered if there is an alteration of enzyme activity in this subfamily of isozyme. In male SD rats the main hepatic isozyme present is CYP2C11 which shares most of its substrates with those metabolized by human CYP3A4 [66].

Testosterone metabolism in human is different from that in the rat. In human, testosterone is metabolized by steroid 5α-reductases in target tissues to the more active metabolite, dihydrotestosterone. Dihydrotestosterone itself is converted in the liver to androsterone, androstanedione, and androstanediol. In rat, CYP2C11 preferentially hydroxylates testosterone in positions of 16α and 2α [63], accounting for 60% of the mass balance. 6β-Hydroxylation of testosterone is catalyzed by CYP3A, whereas 7α-hydroxylation of testosterone is catalyzed by CYP2A1, and 16β-hydroxylation of testosterone is catalyzed by CYP2B1 [68] (see Figure 1.1). So testosterone was chosen

as a probe drug to monitor CYP2C11 the major isoenzyme, activities of the important isoenzymes such as CYP3A, CYP2A1 and CYP2B1 (which also catalyze the testosterone metabolism) can also be monitored, providing more metabolic information in these pathways in the rat.

1.6 RATIONALE FOR CHOOSING NIFEDIPINE AS A MODEL DRUG FOR *IN VIVO* STUDY

Nifedipine metabolism in rat involves oxidative dehydrogenation to dehydronifedipine (M-1), followed by formation of ester cleavage metabolites (M-2 and M-3b) and hydroxylation to the hydroxymethyl metabolite (M-3a). Of these four metabolites, M-1 is the first metabolite, the formation of which is catalyzed by CYP2C11 in SD male rats [69]. The other three are secondary metabolites of M-1 (Figure 1.2). Overall, there are 14 detectable metabolites reported in male rats [70]; other metabolites are metabolic products of these four major metabolites. We postulate that there may be a change in CYP2C11 activity in malnourishment and PN. Nifedipine was chosen as the probe because it is a substrate for CYP2C11 in rats and CYP3A4 in humans.

1.7 PARENTERAL NUTRITION (PN) AND DRUG METABOLISM

The effects of nutrient-drug interactions on the pharmacokinetics of drugs is well documented [71]. PN infusion may cause an instant elevation of the concentration of circulating nutrients in the blood stream. These high concentrations of nutrients may

compete with drugs for plasma protein binding sites and metabolic enzyme sites. As a consequence of competition, drug metabolism could be affected by PN infusion.

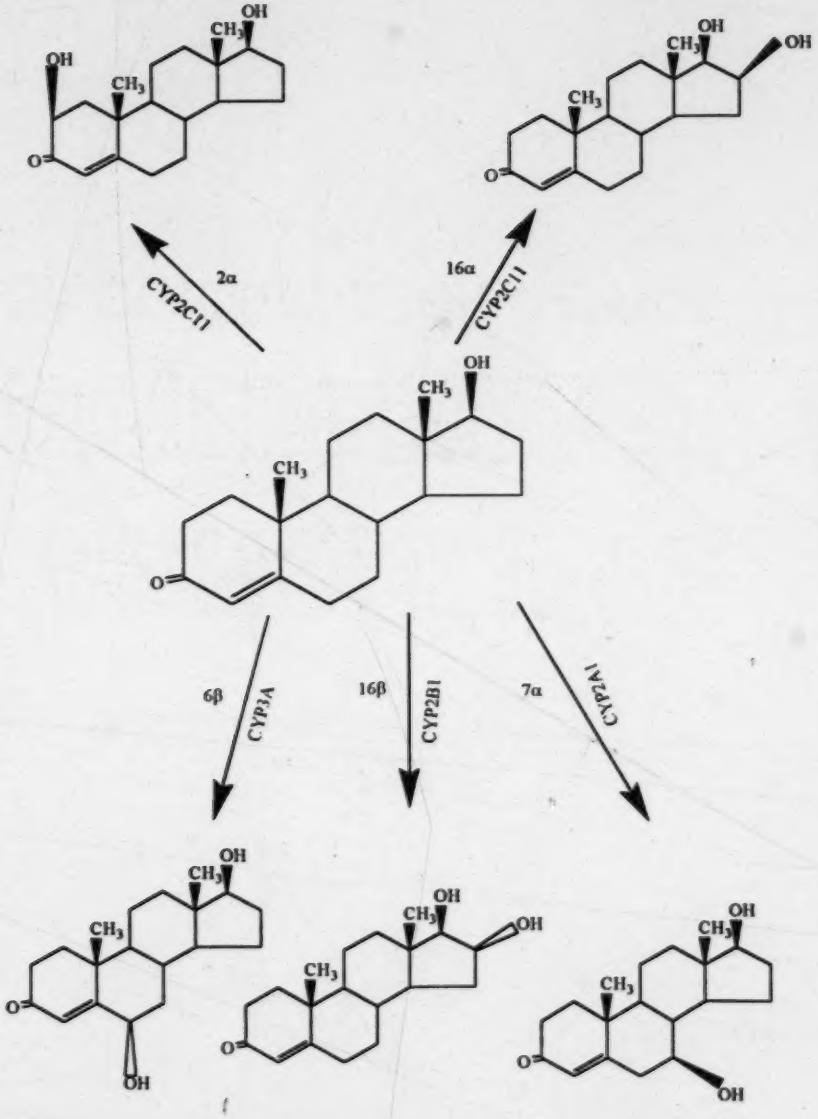

Figure 1.1 Testosterone metabolism scheme in rats [91]

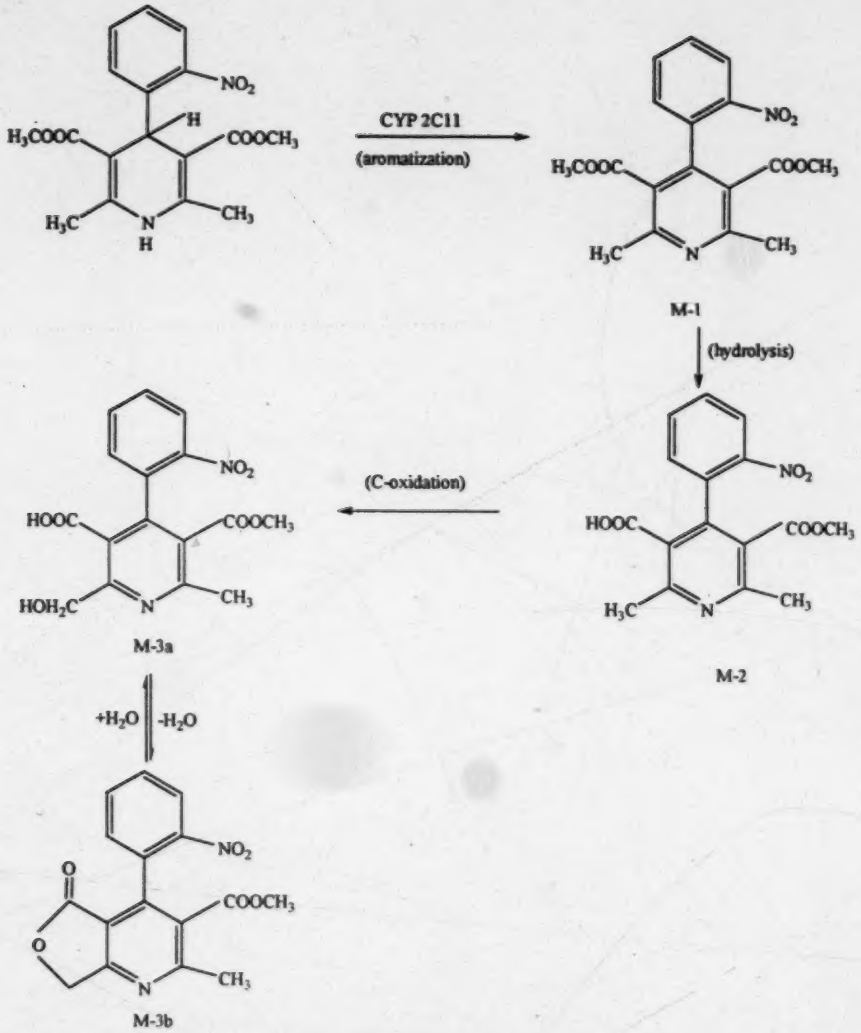

Figure 1.2 Nifedipine metabolism in rats

1.8 RAT GROWTH HORMONE (RGH) AND DRUG METABOLISM

1.8.1 rGH

Growth Hormone (GH), a polypeptide hormone with a molecular weight of 23,000 and released from somatotropes of the anterior pituitary, is regulated by several neurotransmitters and neuropeptides. Among other functions it plays an essential role in regulating body growth and hepatic drug metabolism. In male rats, GH is secreted in episodic bursts every 3.5 to 4 hr. Between the peaks, GH levels are undetectable. In female rats, the hormone peaks are of lower magnitude than in males and occur irregularly, whereas the levels of GH in the troughs between peaks are higher in females [76]. It is not GH *per se*, but its pattern of secretion that regulates the hormone's myriad functions, especially for drug metabolism [77]. Apparently, exposure to the more tonic feminine pattern of GH secretion produces lower growth rates and the lower level of hepatic drug metabolic enzymes found in female rats. Conversely, the circadian rhythm in GH secretion characterized as masculine enhances body growth and allows for the occurrence of a 3 to 5 fold higher levels of hepatic metabolism of many drugs and environmental chemicals [78,79].

1.8.2 rGH and PCM

It is well known that GH and the pituitary-thyroid axis are sensitive to protein-calorie deficit [80-82]. Ventura [83] reported that protein-calorie deficit *per se* may alter

the activity of the pituitary-adrenal axis. Armario et al. [84] conducted a detailed study on chronic food restriction and the circadian rhythms of pituitary-adrenal hormones, growth hormone and thyroid-stimulating hormone. They reported that rats receiving a 65% of the control ration for 34 days resulted in a marked decrease in body, pituitary and adrenal weights. However the weight of both pituitary and adrenals was proportionately higher in food-restricted rats than in the control group. Control rats exhibited a clearly significant difference in serum GH profile. The fluctuation of serum GH in control rats was characterized by two peaks occurring at 11.0 am (around 140 ng/ml) and 8.00 pm (80 ng/ml). Food restriction resulted in marked suppression of the pulsatile GH release although the circadian fluctuations were still significant (fluctuated around 12 ng/ml). The effect of food restriction on GH secretion might be the result of decreased pituitary GH content [82]; alternatively, it might be the result of an inhibition of GH secretion. The finding that passive immunization of starved rats with antisomatostatin serum resulted in the restoration of the amplitude of GH pulses [85,86] suggested that suppression of GH pulses in food restricted animals may also be mediated by somatostatin.

However, Jaya [87] reported that concentrations of plasma GH were higher in kwashiorkor patients than in control people, and normal in marasmus patients. Circulating levels of somatomedin are decreased in patients with kwashiorkor, but are normal in patients with marasmus. Moberg et al. [88] studied the effect of meal feeding on daily rhythms of plasma rGH secretion and found that the effects of food restriction on GH

levels were slight and transient. The effect of malnutrition on rGH release requires further study to confirm this observation.

1.8.3 rGH and Drug Metabolism

The constitutive male-specific CYP2C11 expression in rat liver is developmentally up-regulated at the transcriptional level by the male pattern of GH secretion [79]. The female GH secretory pattern suppresses hepatic CYP2C11 expression and positively regulates hepatic CYP2C12 expression [89-91]. Chen et al. [63] used the hypophysectomized (Hx) rat as a model to evaluate hormonal regulation of CYP2C11 in liver and kidney. Hypophysectomy of male rats led to decreased hepatic CYP2C11 apoprotein and mRNA levels. Twice-daily subcutaneous rGH injections were used, assuming this mode of rGH administration sufficiently approximated the pulsatile GH profile of male rats (high-amplitude GH pulses separated by nadirs of nearly undetectable GH) to restore CYP2C11 to control levels [92]. Similarly, they continuously infused GH to approximate the female rat serum GH profile. The twice-daily GH injections restored CYP2C11 apoprotein and mRNA levels of Hx rats to normal male levels and the GH infusion further reduced the CYP2C11 levels of Hx rats towards female values.

Using a cloned cDNA probe, Janeczko and his colleagues [93] studied the developmental and hormonal regulation of hepatic levels of the mRNA encoding CYP2C. They reported that CYP2C mRNA in the livers of male rats was induced by pulsatile GH secretion. Hypophysectomy led to a 5- to 10-fold reduction in the specific mRNA within

two weeks of the operation, and this effect was reversed by the administration of GH twice daily for 7 days. The changes in CYP2C mRNA levels paralleled those reported for the CYP2C protein by other researchers [90,91], suggesting that pituitary regulation is not exerted at the level of protein turnover. GH levels must, therefore, regulate the transcriptional activity of the CYP2C gene and/or posttranscriptional steps, such as RNA processing, nucleo-cytoplasmic transport, or mRNA stability.

It may be argued that GH can bind to both GH and prolactin receptors of liver and exhibit somatogenic and lactogenic activities [94]. The effects of GH on rat hepatic CYP2C11 and CYP2C12 apoprotein levels have been thought to be mediated through a somatogenic receptor because of the fact that ovine prolactin (a specific lactogen in the rat) had no effects on the levels of CYP2C11 and CYP2C12 apoprotein [95,96]. Rat pituitary extract also restored CYP2C11 apoprotein and mRNA to levels equal to sham controls even when the amount of immunoreactive rGH in the extract was lower than that of purified rGH.

Most studies [63,90,93,96] on the effect of GH on hepatic CYP2C11 used the Hx rat model. The disadvantage of this model is the resultant multiple hormone deficiency that makes identification of specific GH effects difficult, even with hormonal replacement experiments. Thus although all the data from the studies referred to above implicated GH as the primary regulator of hepatic CYP2C11, other pituitary factors or interactions between factors could be involved. Fortunately, Chen and his colleagues [63] studied the effect of anti-rGH serum on hepatic CYP2C11 apoprotein and mRNA levels independent

of other pituitary hormone deficiencies. They used 150 mg lyophilized γ-globulin fraction of sheep anti-rGH serum and injected it subcutaneously to rats twice daily for 11 days. Neutralization of rGH by treatment of intact male rats with sheep anti-rGH serum resulted in decreased mean hepatic CYP2C11 apoprotein level (about 15% of the control), which was accompanied by decreased mean CYP2C11 mRNA levels (about 10% of the control). The effects of anti-rGH serum on CYP2C11 level could not be attributed to neutralization of other pituitary hormones since specificity of this sheep anti-rGH serum has been demonstrated, with cross-reactivity being <0.5% for rat prolactin and rat LH, <0.4% for rat thyroid-stimulating hormone and <0.05% for rat adrenocorticotropic hormone [97]. Therefore, the combined results from other studies of Hx rats [63,90,93,96] and from anti-rGH serum treated rats strongly suggest GH plays a significant role in the regulation of rat hepatic CYP2C11 at the level of mRNA. These results also support the work of Legraverend et al. [79] that demonstrated rGH regulates the transcription of CYP2C11.

1.9 TESTOSTERONE

1.9.1 Pharmacology

Testosterone is a major androgen in the plasma of men. Other androgens are androstenedione, which is a precursor of testosterone, dehydroepiandrosterone (DHEA), and dehydroepiandrosterone sulfate (DHEAS). 95% of testosterone is secreted by the Leydig cells of the testis and 5% is secreted by the adrenal gland. In women, testosterone is synthesized in small amounts by both ovaries and the adrenal gland.

Testosterone serves different functions at different stages of life. During embryonic life, it virilizes the urogenital tract of the male embryo, and its action is thus essential for the development of the male phenotype. The role of testosterone, if any, during the neonatal surge of androgen secretion is not defined but may involve developmental functions within the central nervous system. At puberty, testosterone acts to transform the boy into a man; the penis and scrotum begin to grow, and pubic hair appears. Growth of the larynx and facial hair and the appearance of other secondary sex characteristics are accompanied by body growth since the growth-promoting and anabolic properties of testosterone cause increases in height and the development of the skeletal musculature. For adult male, normal plasma testosterone level (10 to 35 nM) is essential for fertility. It is suggested that testosterone may be responsible in part for the aggressive and sexual behavior of males [98,99]. Therapeutically it is used for hypogonadism as a hormone replacement therapy and as a growth stimulator for delayed puberty. Testosterone is used occasionally in females for the treatment of certain gynecologic disorders such as reducing breast engorgement during the postpartum period, eliminating the endometrial bleeding in the postmenopausal period or in chemotherapy of breast tumors in premenopausal women. In all these cases, testosterone is given in combination with estrogens. Testosterone is also used as a protein anabolic agent in conjunction with dietary measures and exercises in an attempt to reverse protein loss after trauma and surgery or prolonged immobilization, and in patients with debilitating diseases. Large doses of testosterone have been employed in the treatment of refractory anemias and have resulted in some increase in reticulocytosis and hemoglobin levels. Testosterone also has

been used in the treatment of osteoporosis, either alone or in conjunction with estrogen. Testosterone may be used in males of short stature since androgens are responsible for the growth spurt that occurs during adolescence. In this case, the drug should be used with caution since it is also responsible for the eventual termination of linear growth that results from fusion of the epiphyseal growth centers [100].

1.9.2 Chemistry

Testosterone is a naturally occurring androgenic anabolic steroid hormone. The drug may be obtained from animal testes but is usually prepared synthetically from cholesterol. Testosterone is commercially available as cypionate, enanthate and propionate esters.

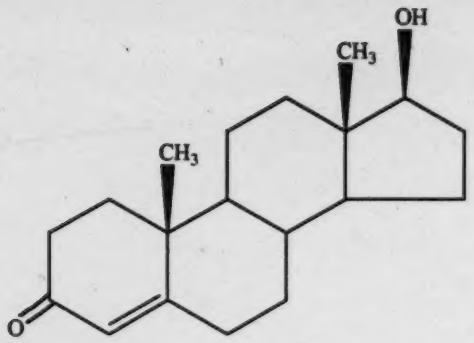

Figure 1.2 Structure of testosterone

Testosterone occurs as white or slightly creamy white, odorless crystals or as a crystalline powder and is practically insoluble in water, freely soluble in dehydrated alcohol, and soluble in vegetable oils.

1.9.3 Pharmacokinetics

1.9.3.1 Absorption

Testosterone injected intramuscularly as a solution in oil is quickly absorbed, metabolized, and excreted so that the androgenic effect is small. Testosterone given by mouth is readily absorbed but is less effective in that most of the hormone is absorbed into the portal circulation and metabolized by the liver before reaching the systemic circulation. Following oral administration of testosterone, only small amounts of the drug reach systemic circulation unchanged. The low bioavailability of orally administrated

testosterone results from metabolism of the drug in the GI mucosa during absorption and on first pass through the liver. Esterification of testosterone generally results in less polar compounds and improves the bioavailability. The cypionate and enanthate esters of testosterone are absorbed slowly into the lipid tissue phase at the IM injection site, so the esters have a prolonged duration of action (up to 2-4 weeks) following IM administration. The duration of action of subcutaneous testosterone pellets is usually 3-4 months, approximately 33, 25 and 17% of testosterone is absorbed within 1, 2 and 3 months, respectively, following subcutaneous implantation [28].

1.9.3.2 Distribution

In plasma, testosterone is 98% bound to a specific testosterone-estradiol binding globulin, and 2% is free. Generally, the amount of sex-hormone binding globulin in plasma determines the distribution of testosterone between free and bound forms. The free testosterone concentrations determine the drug's half-life.

1.9.3.3 Elimination

The plasma half-life of testosterone in human is reported to range from 10-100 minutes. Testosterone metabolism in human is different from the metabolism in rat. In rat liver microsomes, testosterone is mainly metabolized to 16α- and 2α-hydroxy metabolites. 6β-, 7α-, and 16β- hydroxy metabolites are also found (refer to section 1.5). In human, testosterone is metabolized by steroid 5α-reductases in target tissues to the more active metabolite, dihydrotestosterone. Dihydrotestosterone itself is converted in the liver to androsterone, androstanedione, and androstanediol. Androstanedione can be reduced in

the 5β position and can undergo 3-keto reduction to form etiocholanolone [100]. Testosterone can be aromatized to estradiol in a variety of extraglandular tissues and also can be metabolized (hydroxylated) by CYP3A4 at the 6β position [101].

About 90% of a dose of testosterone is excreted in urine as glucuronic and sulfuric acid conjugates of the drug and its metabolites. Approximately 6% of a dose is excreted in feces after undergoing enterohepatic circulation, principally as unconjugated drug [100].

The esters of testosterone are hydrolyzed to free testosterone, but many other changes in the molecule (as in methyltestosterone and fluoxymesterone) alter the course of metabolic degradation. As a result, many synthetic androgens are metabolized less rapidly than testosterone and have longer half-lives.

1.10 NIFEDIPINE

1.10.1 Pharmacology

There are at least four types of voltage-dependent calcium channels: N, P, T, and L [102]. Neurotransmitter secretion is triggered by calcium influx through N or P channels. The T-channels are involved in pacemaker and trigger functions. The L-channels are the predominant type of voltage-dependent calcium channel in the heart and in muscle. This type of channel is of particular importance in the cardiovascular system, where calcium entry through these channels is closely linked to cellular excitation, leading to muscle contraction. The L-type calcium channel is comprised of five subunits: α_1, β,

α_2, γ, and δ [103]. Calcium channel blockers are said to bind with the α_1 subunit of the L-type calcium channels [104,105]. The calcium channel can be present in three different gating patterns: open, closed-responsive and closed-refractory. The effect of drug binding and activity varies considerably depending on prevailing gating patterns.

Calcium channel blockers are divided into 3 major groups: dihydropyridines, phenylalkylamines and benzothiazepines. Nifedipine is a prototype of 1,4-dihydropyridine calcium channel blocker that has high affinity for refractory L and T type channels in resistance vessels. It elicits its therapeutic effect through a potent vasodilator action by blocking the L-type calcium channels in smooth muscle [104-106]. Since 1975, nifedipine has been one of the major cardiovascular drugs used in hypertension and ischemic heart disease in North America [107,108]. It is also indicated for some other uses such as control of unstable angina [109] and long-term prevention of acute myocardial infarction [110].

The mechanism of action of nifedipine is blockage of the influx of calcium, mainly through voltage-dependent L-type calcium channels of cardiac and vascular smooth muscle cell membranes, resulting in inhibition of cell contraction. Unlike verapamil and diltiazem, nifedipine does not suppress calcium influx into the atrioventricular and sinoatrial nodes at therapeutic doses [111]. The potency of nifedipine, as with most 1,4-dihydropyridines, is much greater in smooth than in cardiac muscle, hence the overall haemodynamic effects are dominated by peripheral vasodilation.

1.10.2 Physicochemical Properties

The chemical structure of nifedipine ($C_{17}H_{18}N_2O_6$) is depicted in Figure 1.3

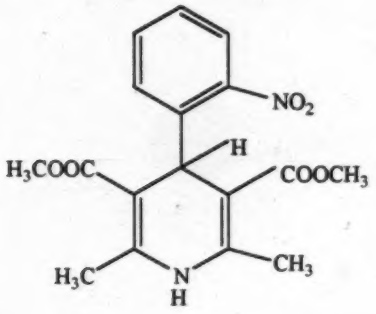

Figure 1.3 Structure of nifedipine

Nifedipine is a yellow, ordorless and tasteless crystalline powder with a molecular weight of 346.3 Dalton [112]. The compound is easily soluble in acetone (250 g/L), methylene chloride (160 g/L) and chloroform (140 g/L), soluble in ethyl acetate (50 g/L), and slightly soluble in methanol (26 g/l) and ethanol (17 g/L). Nifedipine is practically insoluble in water, with a saturated solubility of approximately 10 μg /ml at 37°C, which is reduced to less than 6 μg/ml in phosphate buffer. The n-octanol:water partition coefficient is about 10,000:1 [112]. The melting point is 171 to 175 °C [103]. Nifedipine is a weak base that is not charged at physiologic pH [113]. The ultraviolet (UV) spectrum of nifedipine shows absorption maxima at 235 nm and about 340 nm in methanol solution, and at 238 nm and about 340 nm in alkaline and acidic solutions [112]. Nifedipine is

extremely photosensitive. Exposure of nifedipine to visible and UV light, high temperature or oxidizing agents will yield inactive compounds. Hence, all experimental work with nifedipine should be performed under yellow (sodium) or red light to prevent degradation [114].

1.10.3 Pharmacokinetics

The bioavailability of nifedipine is formulation-dependent. It ranges from 0.40-0.64 in both rat and human [115-117]. Pre-systemic elimination occurs in the gastrointestinal tract and liver [70]. In human, nifedipine is mainly metabolized by oxidative processes *via* the CYP3A4 isoenzyme [69,118]. In rat, more than 90% of a nifedipine dose is metabolized to inactive metabolites following oral administration and more than 80% is *via* the CYP2C11 isoenzyme [70]. The systemic clearance (Clsys) is reported to range from 2.4-10 ml/min/kg, indicative of a low to medium extracted drug, corroborated by a low to medium hepatic extraction ratio (ER_H) (0.22-0.47) [117,118]. Albumin, α_1-acid glycoprotein (AAG) and lipoproteins contribute to the high nifedipine plasma binding (91%-99%) [119,120].

1.11 HYPOTHESES

This project aims at testing the following hypotheses:

1. Malnutrition and PN will result in a decrease in microsomal protein levels and the total P450.

2. Malnutrition and PN will result in an impairment of the activities of CYP2C11, CYP3A1, CYP2B1 and CYP2A1.

3. PN's influence on drug metabolism will be more pronounced in malnourished rats.

4. Nifedipine elimination will be decreased in malnourished adult rats.

5. Nifedipine metabolism will be further impaired in malnourished adult rats after PN treatment.

6. The decrease of rGH is one of the mechanisms that cause the impairment of drug metabolism in malnourished rats.

1.12 RESEARCH OBJECTIVES

The objectives of this project are to use two probe drugs, testosterone and nifedipine to study the effect of protein-calorie malnutrition on drug metabolism and PN's effects on drug metabolism in malnourished rats. The specific objectives of this project are as follow:

1. To establish a malnourished rat model.

2. To evaluate the malnourishment effect on rat liver microsomal protein, total P450 and main metabolic pathways of testosterone in rat liver microsomes for both pediatric and adult rats.

3. To evaluate the malnourishment effect on nifedipine metabolism in adult rats through *in vivo* study.

4. To evaluate the PN effect on rat liver microsomal protein, total P450 and main metabolic pathways of testosterone in rat liver microsomes.

5. To evaluate the PN effect on nifedipine metabolism in malnourished adult rats through *in vivo* study.

6. To monitor rGH's change in malnourished rats.

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CHAPTER 2

A SIMPLE AND SENSITIVE HPLC PROCEDURE FOR SEPARATION AND QUANTIFICATION OF TESTOSTERONE AND ITS METABOLITES IN RATS

2.1 INTRODUCTION

Liver microsomes contain multiple forms of P450 that catalyze the oxidation of innumerable xenobiotics [1,2]. These isozymic hemoproteins determine the duration of action of many drugs and play a key role in chemical carcinogenesis and toxicity [3].

One of the obstacles in the identification of different P450 isoenzymes involved in the metabolism of drugs is the lack of specific and simple enzymatic assays to distinguish the individual isoforms [4]. The regio- and stereospecific hydroxylation of the steroid nucleus is, however, a sensitive fingerprint for the indentification of a specific P450 isoenzyme [5]. In rat, CYP2A1 metabolizes testosterone mainly to 7α-hydroxytestosterone, whereas CYP2B1 forms 16β-hydroxytestosterone and androstenedione [6], and CYP3A forms predominantly 6β-hydroxytestosterone [5], and CYP2C11, the major isoenzyme in male rats, catalyzes the formation of 2α-, 16α-hydroxytestosterone [7]. Therefore, the metabolism of testosterone has been used to probe *in vitro* preparations of rat liver for P450 isoenzyme activities.

Due to the unique catalytic functions ascribed to rat liver microsomes for testosterone metabolism, there is an ongoing need to establish a simple and sensitive

chromatographic method to analyze the hydroxylated metabolites of testosterone. Several chromatographic procedures have been developed for this need [4,5,8-10]. However, some of them require complex gradient systems, and some of them are complicated by significant changes in baseline that accompany the solvent gradient, which affects resolution and detection of later eluting peaks. Some of them do not adequately resolve certain hydroxylated metabolites and require long retention times to separate the metabolites. The objective of this study was to develop a simple, sensitive and short retention time HPLC method for the separation of testosterone and its metabolites in rat liver microsomes.

2.2 EXPERIMENTAL

2.2.1 Chemicals

Testosterone, 6β-hydroxytestosterone, 16β-hydroxytestosterone, 2α-hydroxytestosterone, 7α-hydroxytestosterone, 16α-hydroxytestosterone and cortexolone were purchased from Steraloids Inc. (Newport, Rhode Island 02840, U.S.A.). Acetonitrile and methanol (HPLC grade) were purchased from Fisher Scientific (Nepean, Ontario Canada). Methylene chloride was purchased from Sigma (St. Louis MO USA).

2.2.2 Microsomal Incubation and Extraction

A final volume of 500 µl microsomal reaction mixture containing 250 µM of testosterone, 5 mM $MgCl_2$, 5 µM $MnCl_2$, and 1 mM NADPH in 100 mM potassium phosphate buffer (pH7.4) was pre-incubated at 37°C for 5 min. The reaction was initiated by adding microsomal protein (1mg/ml) to the mixture and carried out in air in microcentrifuge tubes (Brinkmann Instruments Inc., Westbury, NY, U.S.A.) at 37°C. The reaction was terminated after 15 min of incubation by the addition of 6 ml of methylene chloride, then 1 nmol of cortexolone was added as an internal standard. The sample was vortexed on a vortex shaker (IKA-VIBRAX-VXR, Terochem, setting at 1200) for 15 min and centrifuged (1000 *g*) for 10 min, and the methylene chloride layer was removed and dried in a speed vacuum evaporator (Speedvac SC100, Franklin electric USA). The residues were reconstituted in 80µl of acetonitrile and 50 µl of water, and 100 µl of the solution was injected into a HPLC.

2.2.3 HPLC Apparatus and Procedure

The HPLC system (Shimadzu, Tokyo, Japan) was equipped with a LC-600 pump, a SIL-9A auto injector, a SPD -6AV UV detector set at 247 nm and an IBM – compatible PC equipped with Baseline software program (Waters, Milford, MA, USA). The column used was LiChrospher 60 RP-select B C_8 5 µm id, 125× 4 mm (Merck, Darmstadt, F. R., Germany).

The separation system used a simple gradient of mobile phase A (23% of acetonitrile and 77% water) and mobile phase B (100% of acetonitrile). Mobile phase A was used for the first 8 minutes, between 8-18 min, mobile phase B was programmed to increase linearly from 0 to 100%, the proportion of B was reduced linearly to 0 from 18-22 min, and this condition was held for a 2 min equilibration. The flow rate used was 2ml/min.

2.2.4 Recovery, Quantitation and Accuracy

The extraction efficiency (recovery) of testosterone and its metabolites using the sample preparation procedure was tested by spiking authentic metabolites and testosterone standards with concentrations ranging from 0.2 nmol/ml to 5 nmol/ml into the microsomal incubation system without NADPH. Solutions were extracted with 6 ml methylene chloride.

Calibration curves were obtained by spiking standards containing 1–5 nmol/ml each of testosterone and its five metabolites in a microsomal system that has no NADPH. A 1 nmol quantity of cortexolone (10 µl of 10 nmol in 100 ml methol) of was added as an internal standard. Standard curves were constructed by plotting peak-area ratios of testosterone and its five metabolites against their concentrations.

The precision and accuracy of the method were determined by repeated analysis of spiked testosterone and its metabolites with concentrations ranging from the lowest to the highest in the calibration curve. Six replicates of each concentration were analyzed on

three different days. Within-day and day-to-day precision was expressed as coefficient of variation (C.V., %). Day-to-day accuracy was determined by calculating the difference between the mean observed value and the theoretical value as a function of the theoretical value, and the relative error (R.E.) was expressed as a percentage.

2.3 RESULTS AND DISCUSSION

Figure 2.1 shows a representative separation of commercially available testosterone, its metabolites and cortexolone (internal standard). Their retention times are presented in Table 2.1. For all analysis, the wavelength used was 247 nm, which is the maximum absorbance of the α-keto group of the steroid. Some other reports used 254 nm for detection, which is the routinely used wavelength for general steroid analysis, but absorbance is lower at this wavelength [11].

11β-Hydroxytestosterone was used as internal standard in some studies [5]. It should be a good internal standard because 11β-hydroxytestosterone is chemically similar to testosterone metabolites. However, it is expensive and hard to get, and this prohibits routine use. Cortexolone is chemically related to hydroxytestosterone and has the same maximum absorbance at 247 nm. Because cortexolone is cheaper and easier to obtain, cortexolone was used in this study as internal standard.

The distinguishing characteristics of the chromatographic separation illustrated in Figure 2.1 are that the retention time is short and the baseline remains flat throughout the entire run. Run time was long in other assays since the first peak was not eluted until 18

minutes or testosterone was not eluted until 29 minutes later [9,11]. Baseline change was also a problem in other HPLC assays [4,7,10,12], because of their gradient systems. In addition, the use of a low concentration of acetonitrile in the early elution in this study increases the accuracy and provides better resolution. The overall run time is shorter than for all other methods reported in literature {24 minutes vs. 35 minutes or longer [8,10]}. The separation of testosterone and its metabolites produced in a rat liver microsome preparation is shown in Figure 2.2. The results demonstrate further that there was no baseline interference associated with microsomal incubation and chromatography run time was shorter.

All recoveries of testosterone and its metabolites at incubate concentrations of 0.2 and 5nmol/ml were greater than 95% (Table 2.2). The linearity of the method was established over the concentration range 0.2-5 nmol/ml for testosterone and its metabolites. Correlation coefficients were greater than 0.99 for all the compounds measured.

The within-day precision (repeatability) for the analysis of testosterone and its metabolites is shown in Table 2.3. The values were less than 5% for all concentrations studied. The day-to day C.V. (reproducibility) was less than 5% for testosterone and its metabolites at the concentrations investigated. Day-to-day accuracy, expressed as the relative error (R.E.), is shown in Table 2.4. R.E values for all the compounds measured were less than 5% for both concentrations.

In previously published HPLC methods [4,7,9-12], the separation and quatitation of testosterone and its metabolites in rat liver microsome always required a long run time [11], and some did not adequately resolve certain hydroxylated metabolites [6,8,10]. Some of aasays are complicated by significant changes in baseline that accompany the solvent gradient, which affects resolution and detection of later eluting peaks [6,11,8], and some of them required complex gradient systems [11]. The sensitivity of the assay reported is also better than that of the previous studies [11] (The limit of detection is 1 pmol on-column with a limit of quantitation of 3 pmol on-column). This study provides a simple, sensitive and relatively rapid HPLC method for the separation of testosterone and its metabolites produced by rat liver microsomes.

In the literature [4,7,9-12], a C_{18} column was usually used for separating testosterone and its metabolites. Since there is a wide variety of C_{18} stationary phases, complex solvent systems of various combinations of methanol, acetonitrile and tetrahydrofuran were used. The use of a LiChrospher 60 RP C_8 column and our new mobile phase system allow separation of metabolites without the use of tetrahydrofuran or any buffer system.

2.4 CONCLUSION

In conclusion, the HPLC method developed in this study is simple and sensitive for the resolution and quantification of testosterone metabolites produced by rat liver microsomes. Using a LiChrospher 60 RP-select B C_8 column and a simple gradient

mobile phase system, all peaks were well separated and a flat baseline was maintained until all the peaks of interest are eluted. The procedure not only improved the resolution and quatitation of the major testosterone hydroxy metabolites formed by a rat hepatic microsomal system but also significantly shortened the retention time, which saves a significant amount of time in routine sample analysis. The developed method can be used to determine CYP isoenzyme activities in rat hepatic microsomes and has been used to monitor alterations in testosterone metabolism in protein-calorie malnourished and parenteral nutrition-treated rats. This assay can also be used for evaluating hepatic metabolism of new drug candidates and their interactions with other drugs.

2.5 REFERENCE LIST

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Table 2.1 Chromatographic retention times for testosterone and its metabolites in rat liver microsomes

Compound	Retention Time (min)
7α-hydroxytestosterone	3.53
6β-hydroxytestosterone	5.43
16α-hydroxytestosterone	6.51
16β-hydroxytestosterone	9.14
2α-hydroxytestosterone	10.87
Cortexolone	14.75
Testosterone	16.06

Table 2.2 Recovery of testosterone and its metabolites from a microsomal incubation system

Compound	(%) Recovery (mean ± S.D.)	
	0.2 nmol/ml (n=6)	5 nmol/ml (n=6)
7α-hydroxytestosterone	100.5 ± 8.5	95.1 ± 5.1
6β-hydroxytestosterone	101.2 ± 7.8	96.2 ± 4.8
16α-hydroxytestosterone	105.4 ± 6.9	97.5 ± 3.7
16β-hydroxytestosterone	102.8 ± 8.3	95.8 ± 5.5
2α-hydroxytestosterone	104.5 ± 7.4	96.3 ± 4.5
Cortexolone	103.5 ± 2.6	102.8 ± 1.8
Testosterone	100.8 ± 1.1	99.5 ± 2.1

Table 2.3 Within-day analytical precision of testosterone and its metabolites in a microsomal incubation system

Compound	C.V. (%)	
	0.2nmol/ml(n=6)	5nmol/ml(n=6)
7a-hydroxytestosterone	4.1	4.8
6B-hydroxytestosterone	3.8	4.9
16a-hydroxytestosterone	3.5	5.0
16B-hydroxytestosterone	3.6	4.5
2a-hydroxytestosterone	4.9	4.4
Testosterone	2.1	1.2

Table 2.4 Day-to-day accuracy (R.E.) and precision (C.V.) for the analysis of testosterone and its metabolites in a microsomal incubation system

Compound	0.2 nmol/ml (n=6)		5 nmol/ml (n=6)	
	C.V. (%)	R.E. (%)	C.V. (%)	R.E. (%)
7α-hydroxytestosterone	4.6	-3.5	4.5	-4.9
6α-hydroxytestosterone	4.9	4.2	5.0	-4.8
16α-hydroxytestosterone	4.8	4.1	4.9	-3.7
16β-hydroxytestosterone	4.6	-4.3	4.3	-4.6
2α-hydroxytestosterone	4.4	4.1	4.5	-4.5
Testosterone	2.5	-3.5	2.2	-2.1

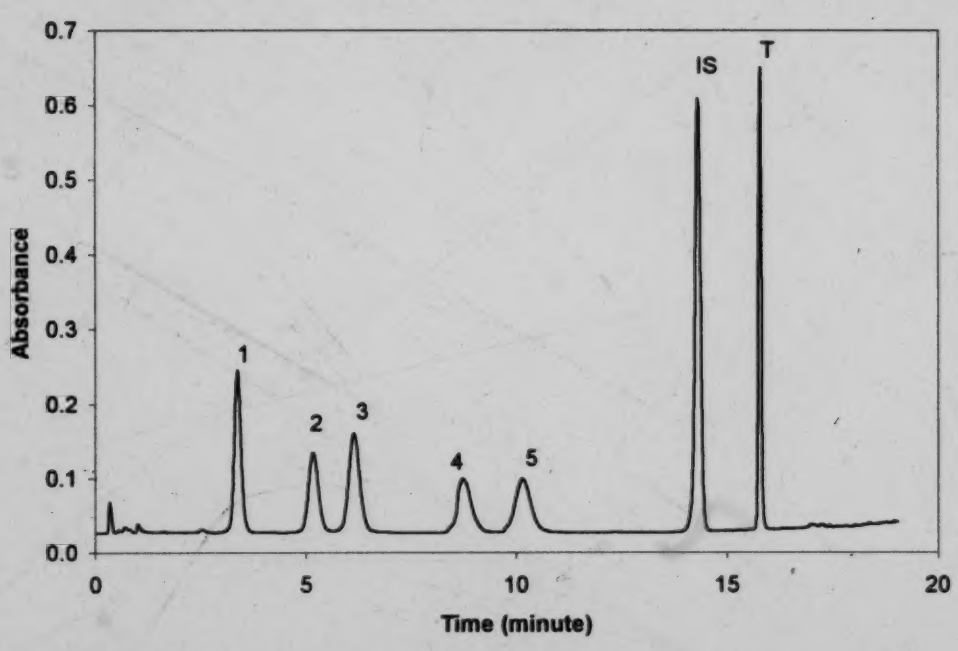

Figure 2.1 Representative chromatogram of commercially available standards at approximately 1 nmol injected on column after extraction (Peaks 1, 2, 3, 4, 5, IS, T are 7α-, 6β-, 16α-, 16β-, 2α-hydroxytestosterone, internal standard and testosterone respectively).

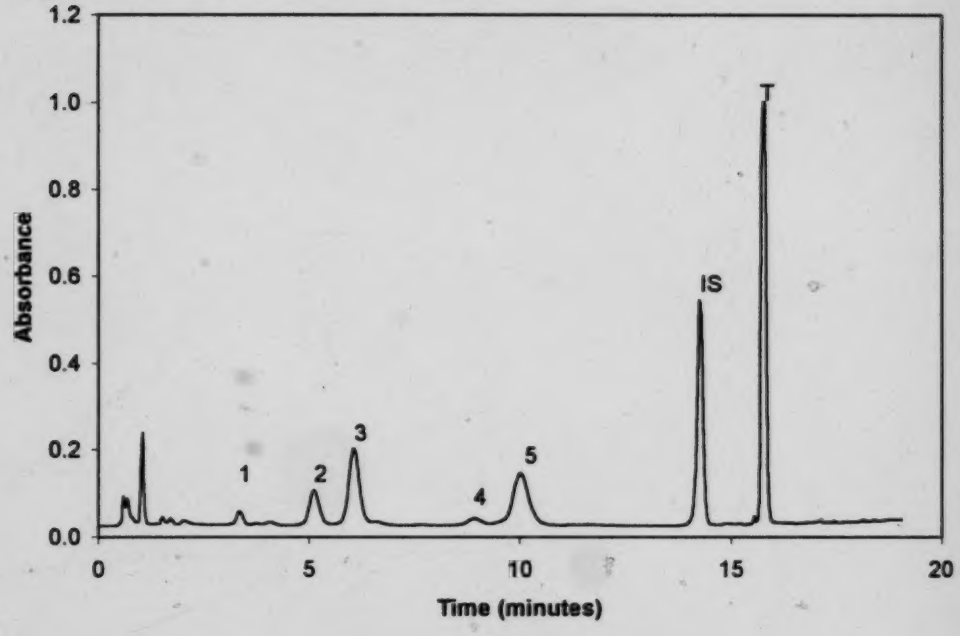

Figure 2.2 Representative chromatograms for testosterone metabolism in liver microsomes from male SD rats. Microsomal protein (1 mg) was incubated with testosterone (250 μM) for 15 min (Peaks 1, 2, 3, 4, 5, IS, T are 7α-, 6β-, 16α-, 16β-, 2α-hydroxytestosterone, internal standard and testosterone respectively).

CHAPTER 3

EFFECT OF PROTEIN-CALORIE MALNUTRITION ON DRUG METABOLISM *IN VITRO*

3.1 INTRODUCTION

The effect of PCM on phase I metabolism is not well characterized in literature. Results in pediatric rats are conflicting [1-3], whereas studies on adult rats are rare. The objectives of this study were to determine the effect of PCM on liver metabolic protein content and total P450, and to investigate protein-calorie malnutrition effects on enzymes such as CYP2C11, CYP3A, CYP2A1 and CYP2B1 by using testosterone as a substrate in both pediatric and adult rats.

3.2 MATERIAL AND METHODS

3.2.1 Chemicals and Reagents

Testosterone, 6β-hydroxytestosterone, 16β-hydroxytestosterone, 2α-hydroxytestosterone, 7α-hydroxytestosterone, 16α-hydroxytestosterone and cortexolone were purchased from Steraloids Inc. (Newport, Rhode Island 02840, U.S.A.). Metofane was from Janssen (North York, Ontario), and halothane was from Halocarbon Laboratories (River Edge, NJ USA). All ingredients of the potassium phosphate buffer (BDH Inc. Toronto, Ontario, Canada) were ACS grade. Glycerol, a reduced form of

NADP and disodium ethylenediaminetetraacetate (EDTA) were purchased from Sigma Chemical Co. (St. Louis MO USA). Bio-Rad reagent was supplied by Bio-Rad Laboratories (Richmond, CA, USA), sodium heparin solution 1,000 i.u./ml by Leo Pharma Inc. (Ajax, Ontario, Canada) and saline by Baxter Co. (Toronto, Ontario, Canada).

3.2.2 Animals and Animal Care

Male SD rats weighing 190-210 g (about 40 days old) were supplied by Biosciences Animal Services, University of Alberta. All animals were housed in the Dentistry-Pharmacy Building Animal Service Facility and were fed Agway (prolab-Formula RMH1000, PMI Feeds, Inc., St. Lois, MO) before starting the experiments. In the initial study, pediatric rats were used (190-210 g, about 40 days old). It was found that pediatric rats were not adequate for malnourishment studies because a lot of stress was created for the animals, which was indicated by the change of hair color to red and the animals were hyperactive. Subsequently, the use of pediatric rats was discontinued and adult rats (340-360 g, about 3 months old) were employed for the study. The protocol for adult rats study was approved by the Health Science Animal Welfare Committee at the University of Alberta.

Pediatric rats: Male SD rats (190-210 g, about 40 days old) were housed individually with a 12-hr light/dark cycle at 21-22 °C. The animals had access to tap water and a nutritionally balanced rat Agway *ad libitum* during an initial 4-day acclimation

period. The feed was weighed daily to determine the amount of food consumed by each rat. After the acclimation period, the animals were randomly divided into two groups (n=5/group). Group 1, the control group, had free access to food and water and Group 2 animals were fed 10 g of food per day and had free access to water. Rats were maintained on the assigned diet for 8-weeks.

Adult rats: Male SD rats (190-210 g about 40 days) had access to tap water and Agway *ad libitum* until the rats were 340-360 g (about three months old). By this time, the rats reached adulthood (three months old). The same protocol for pediatric rats was employed for this study, except that the control animals had 20 g of Agway daily and free access to water to maintain the body weight, while Group 2 animals were fed 12 g of Agway and had free access to water. The assigned diets were maintained for two months.

Assessment of Nutrition Status: Rats were observed for general activity and alertness daily. Serum albumin levels were measured every two weeks and weight measurement was taken twice a week throughout the study period. For adult rats, other than serum albumin, total serum protein was also determined during the experimental period.

Serum Albumin and Globulin Measurements: The tail clip method was used for blood sampling. After rubbing the tip of the tail with a cotton ball soaked with 75% alcohol, approximately 2 mm of the tail was clipped using a sterilized razor blade. Immediately after 50 µl of blood were taken with a capillary tube, silver nitrate sticks were used to stop the bleeding. Blood was centrifuged for 5 min at 2500 g to obtain serum and

samples were frozen at –20°C until analysis. Serum albumin and globulin were determined using direct dye binding with bromocresol green. The measurement was done by the staff at the Surgical Medical Research Institute, University of Alberta.

3.2.3 Preparation of Hepatic Microsomes

After two months of food restriction, SD male rats were fasted overnight and anesthetized with metofane, then the abdominal cavity was opened for portal vein cannulation. The portal vein was cannulated and the liver was perfused *in situ* with ice-cold isotonic (1.15% w/v) potassium chloride solution for about 3 min with a speed of 30 ml/min until the liver turned yellowish (color of liver when blood was cleared). The liver was excised and weighed. The following steps were performed at 4^0C. The livers were finely minced and homogenized in three volumes of ice-cold isotonic potassium chloride solution (1.15% w/v) using a glass homogenizer equipped with a glass pestle (Glas-Col, Cole-Parmer instrument Co., Terre Hautein, IN, USA). The homogenate was centrifuged at 10,000 g for 30 min using a Model IEC B-20A centrifuge equipped with a No. 870 rotor (International Equipment Company, Boston, and MA, USA). The supernatant was evenly distributed into polycarbonate tubes (Ultratube, 13 × 64 mm, Nalge Company, Rochester, NY, USA) and was centrifuged at 105,000 g for 60 min in a model L-55 ultracentrifuge (Beckman Instruments Inc., Palo Alto, CA, USA, rotor type 503 Ti). The microsomal pellet was washed and resuspended in 100 mM potassium phosphate buffer (pH 7.4). After re-centrifugation at 105,000 g for 60 min, the microsomal pellet was resuspended in a volume of 100 mM potassium phosphate buffer, which contains 1 mM

EDTA and 20% v/v glycerol (Sigma Chemical Co., St. Louis, MO, USA). The final volume was equivalent to original liver weight v/w (1 g of liver to make 1 ml microsome) and stored at -80^{0}C in 1 ml aliquots.

3.2.4 Determination of Microsomal Protein Content

Microsomal protein was quantified using the Bradford method [4]. A Standard curve was prepared using bovine plasma gamma globulin (Sigma Diagnostic, St. Louis, Mo, and USA). Bovine plasma gamma globulin was reconstituted with 40 ml double distilled water to obtain a solution of 705 µg/ml. Aliquots of the stock solution (0, 2, 4, 6, 8, 10 and 12 µl) were added in triplicates to the wells of a microtitre plate (Corning Laboratory Sciences Co., Richmond Hill, Ontario, Canada). An appropriate amount of Bio-Rad reagent was added so that the final volume in each well was 200 µl. The original sample taken was 40 µl to determine the protein concentration of liver microsomes, then diluted to a concentration by a factor of 25 to 500. A 10 µl aliquot of each diluted sample was mixed with 190 µl of Bio-Rad reagent (n = 3). Absorbance readings were taken at 590 nm using a Maxline microtitre plate reader (Fisher Scientific, Nepean, Ontario, Canada).

3.2.5 Total Cytochrome P450 Measurement

The Omura and Sato [5] method was used to measure total P450. Microsomes suspended in 0.1 M phosphate buffer, pH 7.4 (2 mg/ml of microsomal protein) were

placed in two 1-cm square cells. One was for sample measurement and the other was for reference. Both cells were carefully bubbled with CO at a rate of one bubble per second for one minute, then the reference cell was covered and to the sample cell was added a few milligrams of sodium dithionite. Subsequently, the sample cell was covered. The difference in spectra of the reference and sample was measured after two minutes. The concentration of total P450 was calculated using Beer's Law with an extinction coefficient of 91 $mM^{-1}cm^{-1}$.

3.2.6 Microsomal Incubation and Extraction

A final volume of 500 µl microsomal reaction mixture containing a 10 µl aliquot of a testosterone stock solution at substrate concentrations of 5, 10, 20, 40, 80, 125, 250, 500, 1000, 2500 µM, 5 mM $MgCl_2$, 5 µM $MnCl_2$, and 1 mM NADPH in 100 mM potassium phosphate buffer (pH7.4) was pre-incubated at 37°C for 5 min. The reaction was initiated by adding microsomal protein (1mg/ml) to the mixture and carried out in air in microcentrifuge tubes (Brinkmann Instruments Inc., Westbury, NY, U.S.A) at 37°C. The reaction was terminated by the addition of 6 ml of methylene chloride after 15 min of incubation, then 1 nmol of cortexolone was added as an internal standard. The sample was vortexed on a vortex shaker (IKA-VIBRAX-VXR, Terrochem, setting at 1200) for 15 min and centrifuged (1000 *g*) for 10 min, and the methylene chloride layer was removed and dried in a speed vacuum evaporator (Speedvac SC100, Franklin Electric

USA). The residues were reconstituted in 80 µl of acetonitrile and 50 µl of water. A 100 µl of reconstituted solution was injected into HPLC.

3.2.7 HPLC Apparatus and Procedure for Testosterone

The HPLC system (Shimadzu, Tokyo, Japan) was equipped with a LC-600 pump, an SIL-9A auto injector, an SPD-6AV UV detector set at 247 nm and an IBM - compatible PC computer system equipped with the Baseline software program (Waters, Milford, MA, USA). The column used was LiChrospher 60 RP-select B C_8 5 µm id, 125 × 4 mm (Merck, Darmstadt, F. R., Germany).

The separation system consisted of mobile phase A (23% of acetonitrile and 77% water) and mobile phase B (100% of acetonitrile). A gradient system started with 100% of A. Between 8-18 min, mobile phase B was programmed to increase linearly from 0 to 100%, and the proportion of B was reduced linearly to 0 from 18-22 min; this condition was held for two minutes for equilibration. The flow rate used was 2 ml/min.

3.2.8 Statistic Analysis

One way analysis of variance (ANOVA) and the Student t-test were used to analyze all the parameters measured in malnourished and control rats. The level of statistical significance of the parameters between the experimental and control groups was identified using the ANOVA analysis. A value of $P < 0.05$ was considered statistically significant and the power of the test is set at 80%. It is discovered that using 5 or 6

animals in each study group, the power of the test as calculated uing the equation $n = (z\alpha + z\beta)^2 * 2\sigma^2/\delta^2)$ is higher than 90% in all cases. Data are expressed as mean ± SD, unless stated otherwise.

3.3 RESULTS AND DISCUSSION

Table 3.1 shows that during two months of food restriction, serum albumin levels did not change significantly in either experimental or control groups. Table 3.2 and Figure 3.1 show that after two months of food restriction, the body weight, and liver weight of pediatric rats decreased to about 50% of the control values, and microsomal protein and total P450 per gram of liver decreased to about 60% of the control. Table 3.3 shows that, except for 16β-testosterone hydroxylation, the V_{max} values of hydroxylation decreased significantly in PCM pediatric animals compared to those of control, while the K_m values of these enzymes increased significantly. The formation of 2α-hydroxytestosterone and 16α-hydroxytestosterone are catalyzed by CYP2C11 [6] and 6β-hydroxytestosterone formation is catalyzed by CYP3A; 7α-hydroxytestosterone formation is catalyzed by CYP2A1, while 16β-hydroxytestosterone formation is catalyzed by 2B1 [7]. These results suggest that both the capacity and affinity of CYP2C11, CYP2A1 and CYP3A were impaired in PCM pediatric rats while CYP2B1 did not show any significant difference in activity. The results of this study are comparable with those reported in the literature. Previous studies [1,8-10] reported a decrease in the levels of liver DNA and microsomal proteins in rats with protein malnutrition, together with a reduction in P450,

P450 reductase and several other microsomal enzymes. These studies also demonstrated a decrease in CYP activity in conditions of malnutrition. Observations made by Bulusu and Chakravarty [11] showed three weeks of protein deprivation caused significant reductions in the enzyme activities both at 6% and 3% protein levels, and caused the inhibition of aniline hydroxylase and aminopyrine (substrates for CYP2C and CYP3A) demethylase activities. From this study, it seems that serum albumin level is not a good indicator of malnutrition for drug metabolism studies. All the important metabolism parameters such as total P450, microsomal protein, capacity and affinity of most of the enzymes were changed significantly after two months of food restriction, while serum albumin was virtually unchanged. This is interesting since conventionally, serum albumin has been set as a criterion for evaluating animal malnutrition [2,12]. Actually there are two kinds of severe malnutrition: marasmus and kwashiorkor. Marasmus is a calorie malnutrition and is characterized by weight loss, emaciation, loss of turgor in the skin, almost total disappearance of subcutaneous fat and atrophy of the muscles. Plasma albumin concentrations may or may not be changed. Kwashiorkor is a protein malnutrition and manifests as inadequate growth of pediatric subjects, loss of muscular tissue, oedema and hepatomegaly. For kwashiorkor subjects, low serum albumin is often observed [13]. In our study, since the food fed contained 17% of protein, it was not a pure protein malnutrition. Actually, in human malnutrition studies, it was found that serum albumin is a poor parameter for evaluating individual patients' nutritional state in that it does not consistently reflect significant body compositional changes [14].

Table 3.4 and Figure 3.4 give the physical characteristics, serum albumin, total serum protein, microsomal protein and total P450 values after two months of food restriction in adult PCM rats. Body weight of the malnourished rats was about 60% of the controls. This reduction complies with the ethics guidelines of our institute (we were required to keep the malnourished rat body weight no less than 250 g). Since they were adult rats, to keep the control rats from being obese, a maintenance ration of 20 g was given to the control rats, while the malnourished got 12 g of food to keep them around 250 g. The difference in the amount of food given to each rat in the study group was within 0.5 g.

Similar to the change in body weight, liver weight of the malnourished adult rat was about 60% of that of the control. It decreased proportionally to the body weight (Figure 3.4). The decrease of liver weight was not as dramatic as that in pediatric rats (compare 40% decrease in PCM adult rats to 50% decrease in PCM pediatric rats) since the diets were a little bit different and the pediatric rats were in the growing stage.

Since serum albumin levels did not change in PCM pediatric rats, in addition to serum albumin, total serum protein level was also monitored in PCM adult rats. No differences in the serum albumin and total serum protein levels were found between the control and the malnourished rats. The lack of change in the serum protein levels suggests these parameters are not good indicators of malnutrition in drug metabolism studies. Changes in parameters such as total P450, microsomal protein and capacities and affinities of major enzymes better reflect a change in nutritional status.

Figure 3.4 illustrates the differences in body weight, liver weight, total serum protein, microsomal protein, and total P450 in adult malnourished and control groups. From the figure, we can see that microsomal protein in malnourished rats is significantly lower than that of controls, but the decrease was smaller than that observed in pediatric rats. Total P450 in adult malnourished rats was also significantly lower; it was about 70% of that of the control. Compared to the pediatric study in which total P450 was less than 60% of that of the controls, the impairment is not as severe in the malnourished adult rats. The less severe reduction of microsomal protein and total P450 in PCM adult rats might be explained by the slight difference in the diet and by the fact that pediatric rats were still growing. In the pediatric stage, protein synthesis is more sensitive to environmental changes, the immune system is more fragile to foreign insults, and consequently the degree of protein and enzyme reduction is more severe [3].

Table 3.5 shows the values of V_{max} and K_m of 16α-, 2α-, 7α-, 6β- and 16β-hydroxylation of testosterone metabolism pathways in PCM adult rats. Except for 16β-hydroxylation, the V_{max} values of testosterone hydroxylation 16α-, 2α-, 7α- and 6β- were found to be significantly lower in the malnourished group than in the controls. Compared to the V_{max} values of those in pediatric rats, the decrease of V_{max} values of 16α, 2α-, 7α-, 6β-hydroxylation in adult rats was not as dramatic, while the V_{max} of 16β-hydroxylation did decrease, but not significantly. The K_m values of 16α-, 2α-, 7α- and 6β-hydroxylation increased significantly. The changes were similar to those observed in the pediatric rats.

The K_m of adult rat 16β-hydroxylation did not show a significant change. A similar observation was made in the pediatric study.

16α-Hydroxylation of testosterone has a large capacity, since it becomes saturable at very high substrate concentrations (> 2.5 mM for control and >2.0 mM for malnourished) (Figure 3.2, Figure 3.5). Lineweaver-Burk plots are linear (Figure 3.3, Figure 3.6), implicating no substrate or metabolite inhibition or induction.

From the results we can see that PCM impaired the capacities and affinities of CYP2C11, 3A and 2A1 in adult rats, whereas CYP2B1 did not show any significant difference both in capacity and affinity under PCM condition. The impairment of the enzyme capacities in pediatric rats was more severe than that in adult rats. Pediatric rats may be more sensitive to malnutrition.

CYP2C11 is a major isoenzyme in the liver of male rats. This isoenzyme accounts for about one third of the total cytochrome P450 in SD male rat liver [6] and it metabolizes a host of xenobiotics such as benzphetamine, aminopyrine, ethylmorphine, benzopyrene and warfarin [6]. Furthermore, rat CYP2C11 shares most substrates for metabolism with human CYP3A4 [15]. Human CYP3A4 is a major isoenzyme in human liver; this single isoenzyme is involved in about 60% of all oxidations of clinically used drugs, including erythromycin, nifedipine, testosterone, and lidocaine [16]. In addition to CYP2C11 activity, CYP3A and CYP2A1 activities are also impaired significantly by malnutrition. Interestingly, CYP2B1 did not show a significant change. Increases and

decreases of antipyrine metabolism in malnourished subjects have been reported [17-19]. CYP2B1 is one of the major isoenzymes which metabolizes antipyrine in rats [20,21].

In the literature, effects of malnutrition on drug metabolism are conflicting. Anthony [1] reported malnutrition severely impaired drug metabolism. Leakey's [3] group reported caloric restriction decreased the age-related changes in hepatic testosterone metabolism. Catz [2] found that in neonatal animals with malnutrition, the difference in the rate of metabolism was not uniform. For oxidative pathways, an increase was found using aminopyrine and benzpyrene as substrates. However, the rate of aniline metabolism was not altered. For reductive pathways, a decrease was observed with 2 substrates: p-nitrobenzoic acid and neoprontosil. The discrepancy could come from the use of different substrates, the variety of ages of the animal used and the different degrees of malnutrition in these studies. Differently aged animal may respond to malnutrition differently and different degrees of malnutrition may have different effects on drug metabolism. Also, malnutrition could influence each isoenzyme differently. The study reported here employed pediatric and adult rats, and investigated malnutrition's effect on several isoenzymes, including the major one, CYP2C11. Therefore, the results from this study have more practical significance.

Most reported animal studies about malnutrition's effect on drug metabolism were about protein malnutrition in pediatric rats, and few were on protein-calorie malnutrition. No reports have been found reporting a correlation of PCM to drug metabolism for adult

rats. This study clearly showed that the effect of PCM, as a whole (not just pure protein malnutrition), on drug metabolism both in pediatric and adult rats.

3.4 CONCLUSIONS

From this study we conclude that PCM decreases microsomal protein and total P450 levels in both pediatric and adult rats. PCM impairs the abilities of CYP2C11, CYP3A and CYP2A1 to hydrolyze testosterone. CYP2B1 activity did not show any significant change either in capacity or affinity in malnourished pediatric and in malnourished adult rats. Changes in PCM adult rats were not as dramatic as those in pediatric rats. Neither serum albumin nor total serum protein level is a good indicator of malnutrition.

From the results of this study, it is obvious that malnutrition impairs the activity of many CYP isoenzymes, including CYP2C11, a major isoenzyme in male rat liver. If this observation can be extended to humans, then PCM might have a significant effects on altering drug metabolism in human PCM subjects.

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Table 3.1. Average (±SD) levels of serum albumin (g/L) in pediatric rats during two months of food restriction (n=5/group)

Duration	Serum albumin	
	Malnourished	Control
Second week	34.8 ± 3.1	35.9 ± 5.8
Fourth week	34.6 ± 3.8	35.3 ± 3.5
Sixth week	34.1 ± 3.6	35.8 ± 4.8
Eighth week	34.0 ± 2.5	35.3 ± 3.9

Table 3.2 Mean (± SD) physical and microsomal parameters in malnourished pediatric rats after two months of food restriction (n = 5/group)

Parameters	Malnourished	Control
Body weight (g)	230 ± 14*	470 ± 10
Liver weight (g)	7.9 ± 0.9*	16.9 ± 0.5
Serum Albumin (g/L)	34.0 ± 2.5	35.3 ± 3.9
Microsomal Protein (mg/g liver)	25.8 ± 2.6*	43.5 ± 2.5
Total P450 (nmol/g liver)	14.3 ± 3.0*	24.1 ± 3.6

*$p<0.05$ compared with controls

Table 3.3 Mean (± SD) kinetic parameters of testosterone metabolism in liver microsomes of malnourished pediatric rats (n = 5/group)

	V_{max} (pmol/min/mg)		K_m (μM)	
Pathways	Malnourished	Control	Malnourished	Control
16β-hydroxylation	59.1 ± 5.21	63.4 ± 6.3	39.4 ± 4.3	38.9 ± 3.3
7α-hydroxylation	196.4 ± 25.5*	348.4 ± 35.1	16.1 ± 2.7*	9.1 ± 0.76
2α-hydroxylation	2439 ± 225*	5363 ± 525	22.7 ± 2.3*	15.1 ± 1.7
16α-hydroxylation	1538 ± 165*	3448 ± 345	26.6 ± 2.1*	10.3 ± 1.8
6β-hydroxylation	322 ± 35*	833 ± 75	26.3 ± 2.3*	13.5 ± 1.1

*$P < 0.05$ compared with controls

Table 3.4. Mean (± SD) physical and microsomal parameters in malnourished adult rats after two months of food restriction (n = 6/group)

Parameters	Malnourished	Control
Body weight (g)	267 ± 10*	445 ± 27
Liver weight (g)	8.1 ± 0.4*	13.9 ± 1.9
Serum albumin (g/L)	34.0 ± 4.8	34.5 ± 2.1
Total plasma protein (g/L)	65.5 ± 10.1	66.1 ± 8.6
Microsomal Protein (mg/g liver)	27.6 ± 3.2*	40.1 ± 4.1
P450 liver (nmol/g liver)	15.8 ± 2.2*	23.2 ± 2.8

*$p < 0.05$ compared with controls

Table 3.5 Mean (± SD) kinetic parameters of testosterone metabolism in malnourished adult rat liver microsomes after two months of food restriction (n = 6/group)

	V_{max} (pmol/min/mg)		K_m (μM)	
Pathways	**Malnourished**	**Control**	**Malnourished**	**Control**
16β-hydroxylation	58.3 ± 7.2	65.4 ± 10.1	36.5 ± 5.0	35.3 ± 4.3
7α-hydroxylation	217.4 ± 24.3*	396.8 ± 34.2	17.4 ± 2.7*	10.2 ± 2.4
2α-hydroxylation	2941 ± 289*	5263 ± 511.1	23.7 ± 3.1*	16.7 ± 2.7
16α-hydroxylation	1960 ± 210*	2941 ± 299	22.5 ± 2.7*	11.1 ± 2.7
6β-hydroxylation	414 ± 46*	753 ± 66	27.3 ± 2.7*	15.2 ± 1.7

*$P < 0.05$ compared with controls

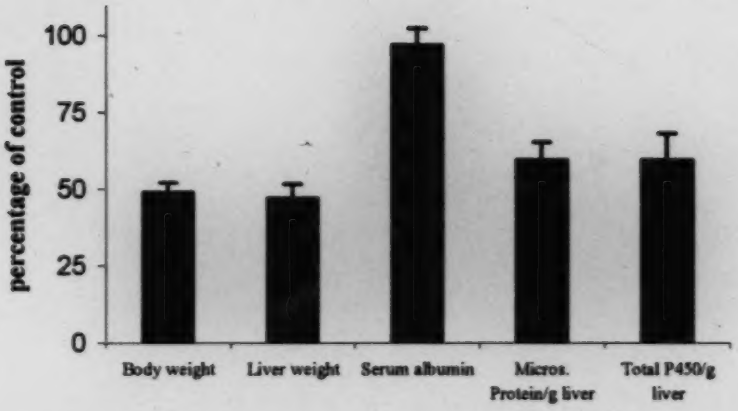

Figure 3.1 Physical and microsomal parameters in PCM pediatric rats expressed as percentage of control after two months of food restriction (n=5/group). Results are expressed as mean ± S.D.

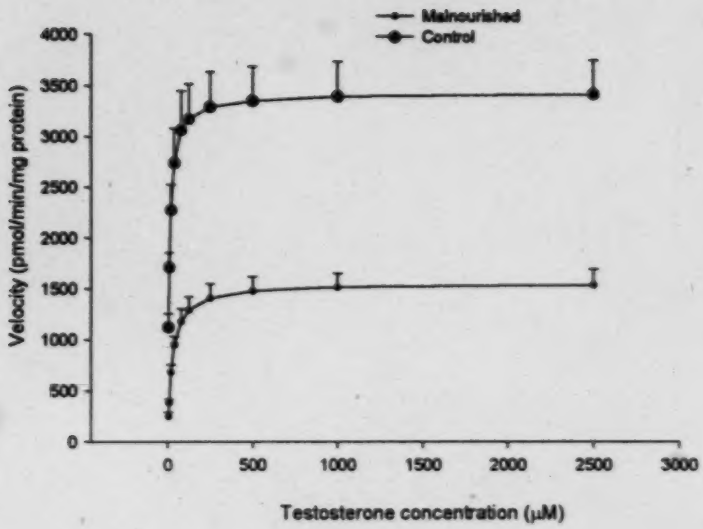

Figure 3.2 Michaelis-Menten plots of 16α-hydroxytestosterone formation in pediatric rat liver microsomes. Substrate concentration ranged from 5-2500 μM. Results are expressed as mean ± S.D.

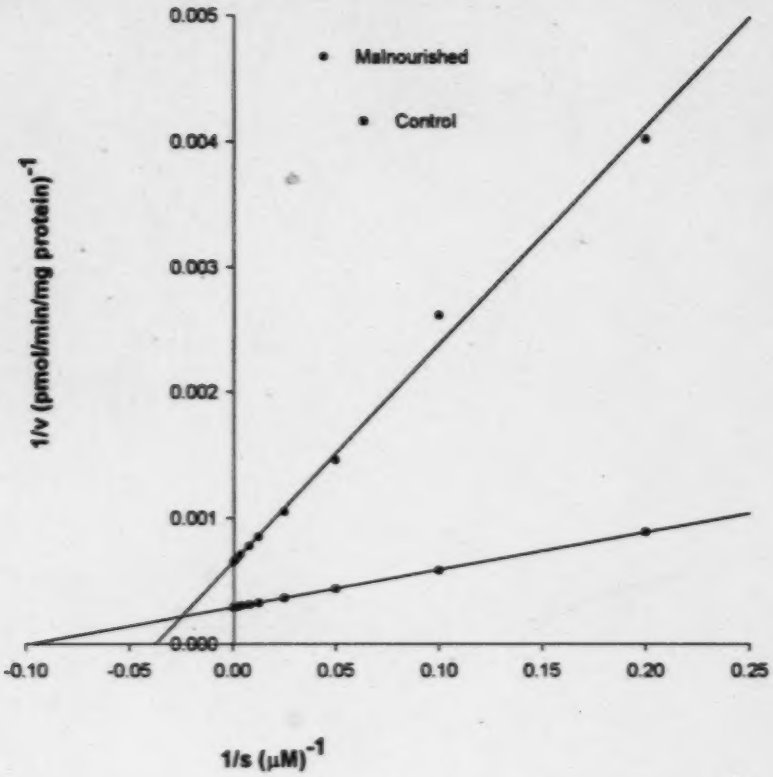

Figure 3.3 Lineweaver-Burk plot of 16α-hydroxytestosterone formation in PCM pediatric rat liver microsomes. Substrate concentrations ranged from 5-2500 μM

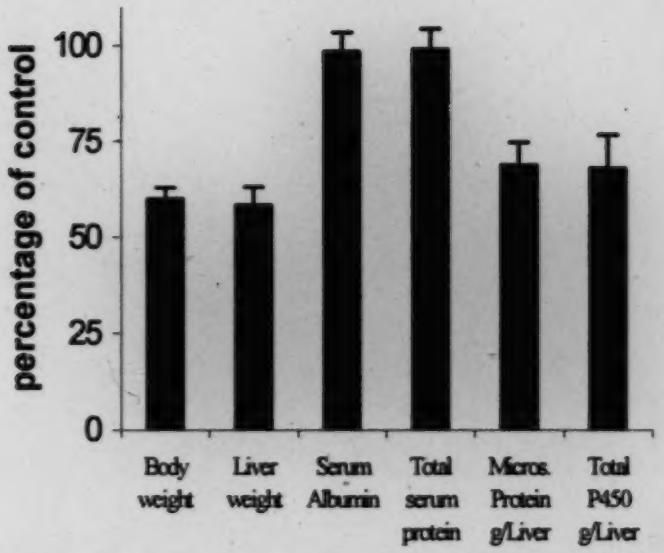

Figure 3.4 Physical and microsomal parameters in PCM adult rats expressed as percentage of control after two months of food restriction (n=6/group). Results are expressed as mean ± S.D.

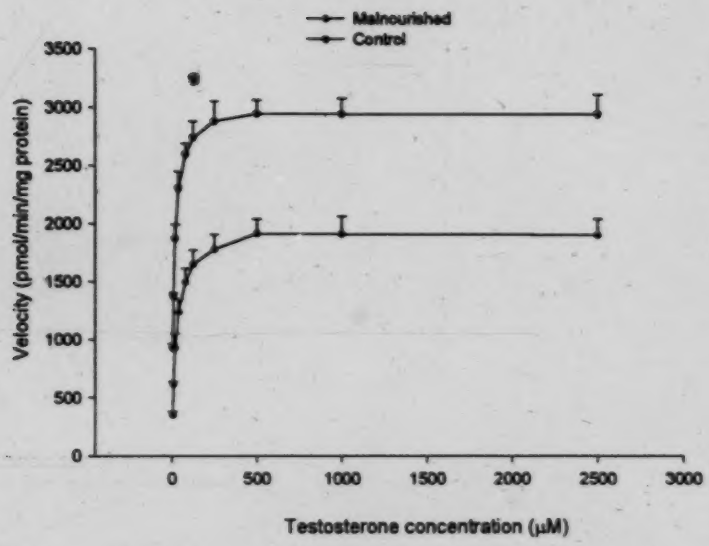

Figure 3.5 Michaelis-Menten plots of 16α-hydroxytestosterone formation in PCM and control adult rat liver microsomes (Testosterone concentration ranged from 15-2500 μM). Results are expressed as mean ± S.D.

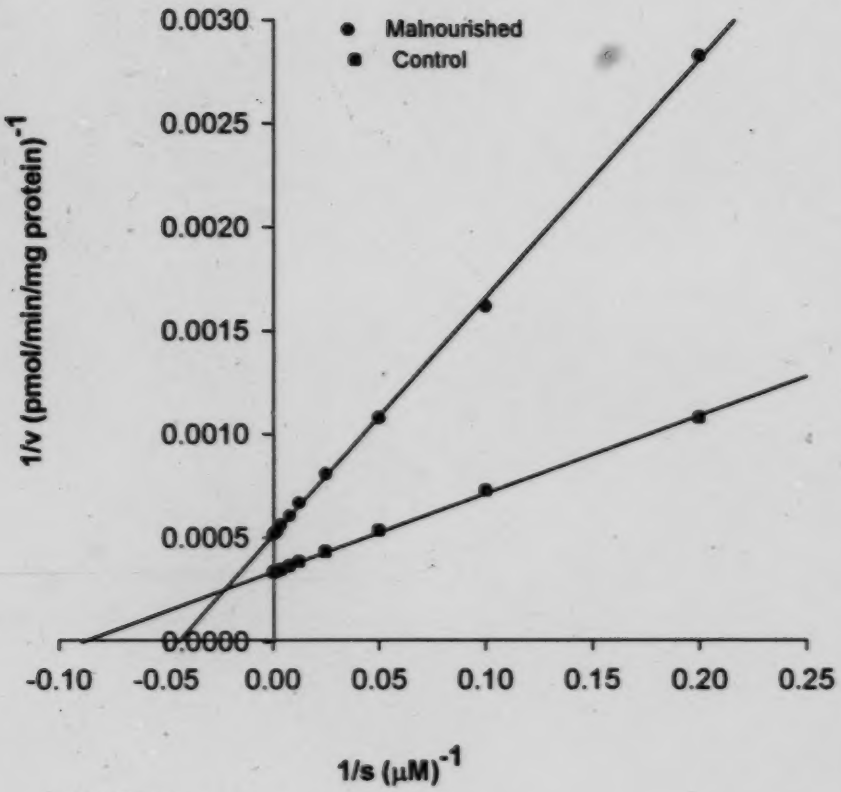

Figure 3.6 Lineweaver-Burk plot of 16α-hydroxytestosterone formation in PCM adult rat liver microsomes. Substrate concentrations ranged from 5-2500 μM

CHAPTER 4

EFFECT OF PROTEIN-CALORIE MALNUTRITION ON DRUG METABOLISM IN ADULT RATS (*IN VIVO*)

4.1 INTRODUCTION

The impairment of protein-calorie malnutrition on drug metabolism in a microsome study in Sprague-Dawley (SD) male rats was demonstrated in our previous study (please refer to chapter 3). The objective of the study described in this chapter was to investigate the effect of protein-calorie malnutrition on nifedipine clearance in adult male rats through an *in vivo* study using nifedipine as a probe drug. Nifedipine has more than fourteen detectable metabolites in rats, but 80% of the metabolites are sequential metabolites of nifedipine's metabolite M-1 (dehydronifedipine) [1]. The conversion of nifedipine to dehydronifedipine is catalyzed by CYP2C11 in SD male rats [2], so the PCM effect on CYP2C11 will be elucidated in this *in vivo* study.

4.2 MATERIAL AND METHODS

4.2.1 Chemicals and Reagents

Nifedipine and nitrendipine were purchased from Sigma Inc. (Mississauga, ON, Canada). Acetonitrile and methanol were HPLC grade and purchased from Fisher Scientific (Nepean, Ontario, Canada). Methylene chloride and isooctane (HPLC grade)

were from BDH (BDH Inc., Toronto, Ontario, Canada), acetic acid, methyl-t-butyl ether (MTBE) and triethylamine (TEA) were analytical grade and were purchased from Fisher Scientific (Nepean, Ontario Canada). Polyethylene glycol (PEG 400) was obtained from Union Carbide Chemicals (Danbury, CT, USA).

4.2.2 Animals and Animal Care

Male SD rats weighing 190-210 g were supplied by Biosciences Animal Services, University of Alberta. All animals were housed in the Dentistry-Pharmacy Building Animal Service Facility and had access to tap water and Agway (Prolab-Formula RMH1000, PMI Feeds, Inc., St. Lois, MO) *ad libitum* until the rats weighed 340-360 g. By this time, the rats had reached adulthood (three months old). Then, 12 healthy male adult SD rats were randomly divided into malnourished group and control group. The control animals received 20 g of Agway daily and had free access to water to maintain body weight, while group 2 animals were fed 12 g Agway and had free access to water. The assigned diets were maintained for two months.

Assessment of Nutrition Status: Rats were observed for general activity and alertness daily. Serum albumin and total serum protein levels were measured every two weeks and weight measurement was taken twice a week throughout the study period.

Serum Albumin and Total Serum Protein Measurements: The tail clip method was used for blood sampling. After rubbing the tip of the tail with a cotton ball soaked with 75% of alcohol, approximately 2 mm of the tail was clipped using a sterilized razor blade.

Immediately after 50 µl of blood were taken with a capillary tube, silver nitrate sticks were used to stop the bleeding. Blood was centrifuged for 5 min. at 2500 g to obtain serum and samples were frozen at –20°C until analyzed. Serum albumin and total serum protein were determined using a direct dye binding method with bromocresol green. The staff at the Surgical Medical Research Institute, University of Alberta, made the measurements.

4.2.3 Dosage Form Preparation

Nifedipine solutions were prepared by adding nifedipine powder to PEG 400 (5mg /ml) the day before administration to allow sufficient time for drug solubilization. Containers were wrapped in aluminum foil to protect the solutions from light and stored at room temperature. To prevent nifedipine degradation, all dosage formulations were prepared and handled in a room with sodium light.

4.2.4 Surgical Cannulation Procedure

Male SD rats after malnutrition treatment were moved to the laboratory and subjected to surgical cannulation. Under anesthesia induced by metofane, a small longitudinal incision was made in the skin of each rat over the right jugular vein. The vein was catheterized with 3 cm of silastic tubing (0.635 mm i.d., 1.194 mm o.d., Dow Corning Corp., Midland, MI USA) that contained heparin zed (80 IU/ml) normal saline and was fixed in place with two no absorbable surgical sutures (Surgical Suture USP, Cyanamid Canada Inc., Montreal, QC, Canada). Each cannula was terminated with a suitable length

of polyethylene tubing, which has an internal and external diameter of 0.58 and 0.97 mm (PE-50, clay Adams, parisppany, NJ, USA). All rats were allowed to recover from surgery for two days. To maintain the potency of the catheter, the catheter was flushed daily and locked with heparinized saline (80 IU/ml).

4.2.5 Nifedipine Administration and Blood Sampling

Nifedipine (5 mg/ml PEG) was given by a 4 min intravenous infusion. The dosage was 6 mg/kg. Before nifedipine administration, about 500 μl blood was taken out for washing the catheter. The dose was injected slowly from 0 to 4 min through the jugular vein cannula. Blood samples of approximately 150–250 μl were drawn through the jugular vein cannula with heparinized-coated 1-ml tuberculin syringes at time 0, 6, 8, 10, 15, 20, 30, 45, 60, 90, 120, 150, 180 and 240 min and the same volume of saline was injected to replace the lost blood. Immediately after blood sampling the cannula was rinsed 3 times, first with about 40 μl blood, then twice with about 0.1 ml saline (the same volume as the blood sample taken). This procedure served as the replacement of lost blood. Each rinse was carried out very slowly to make sure all nifedipine on the tube was washed out (this procedure is necessary to prevent any carryover from drug administration). Each blood sample was centrifuged for 5 min at 2500 g to obtain plasma. Samples in containers were wrapped in aluminum foil and frozen at -20^{0}C until analyzed.

4.2.6 Analysis of Plasma Nifedipine

Plasma nifedipine concentrations were measured by a HPLC method reported by Grundy et al. [3] with some modifications. The internal standard used was nitrendipine. The analytical method is briefly described as follows: a 100 µl of plasma sample was basified with 1.0 M sodium hydroxide, then extracted with methyl-t-butylether (MTBE):iso-octane (75:25, v/v). The resulting organic phase was evaporated under vacuum. The residue was reconstituted with 200 µl of mobile phase (methanol:water:acetic acid:triethylamine, 65:34:1:0.03, v/v). A 100 µl portion was injected onto a Nova-Pak 8 × 100 mm radial pack column containing 4 µm C8 as stationary phase (Waters, Mississauga, ON., Canada). Nifedipine was detected using a SPD–6A UV-Vis detector (Shimadzu Corporation, Tokyo, Japan) set at 250 nm. Calibration curves were linear (r^2 >0.99) from 10 to 2500 ng/ml. All HPLC runs had a total of 9 quality control samples, including 3 each at low (10 ng/ml), medium (200 ng/ml) and high (1000 ng/ml) concentrations. Intra- and inter-day variability was less than 7%. Accuracy and precision were within 10% over the concentration range. Baseline software (Waters, Milford, MA, USA) was used to integrate peaks and process chromatographic data.

4.2.7 Statistic Analysis

One way analysis of variance (ANOVA) and the Student t-test was used to analyze the pharmacokinetic parameters of malnourished rats and controls. The level of statistical significance of the parameters between the experimental and control groups was identified using the ANOVA analysis. A value of $P < 0.05$ was considered statistically significant and the power of the test is set at 80%. It is discovered that using 6 animals in each study group, the power of the test as calculated uing the equation $n = (z\alpha + z\beta)^2 * 2\sigma^2 / \delta^2)$ is higher than 90% in all cases. Data are expressed as mean ± SD, unless stated otherwise.

4.3 RESULTS AND DISCUSSION

After two months of food restriction, the body weight of a malnourished rat was about 60% of the control value. Serum albumin and total serum protein levels did not show any statistical difference between malnourished and control animals (Table 4.1).

The mean (±SD) plasma nifedipine concentration-time profiles obtained in this study after *iv* dosing of nifedipine (6mg/kg) to malnourished and control rats are shown in Figure 4.1. The individual profile obtained after *iv* dosing displayed a distributive phase with multi-exponential decline and a monoexponential elimination phase. Standard pharmacokinetic parameters calculated from the data obtained in this study are shown in Table 4.2. Values of $AUC_{0\text{-last}}$, $AUC_{0\text{-}\infty}$, $t_{1/2}$, CL_{TB} and V_{ss} were found to be significantly different between malnourished rats and control ones. CL_{TB} was much lower in the

malnourished than in the control animals, and consequently $AUC_{0\text{-last}}$, $AUC_{0\text{-}\infty}$, $t_{1/2}$ values were significantly higher in malnourished than in control rats. As suggested by the profiles in Figure 4.1, these data clearly indicate that PCM impaired the elimination of nifedipine in SD male rats.

Little or no unchanged nifedipine is found in the excreta (urine and bile) in rats [1,4], therefore, the drug is cleared from plasma *via* biotransformation, presumably by the liver after *iv* administration. Hence the systemic clearance can be assumed to equal that of hepatic clearance [4]. The mean systemic nifedipine clearance found in this study for control rats (Table 4.2) is similar to that given by Boje et al. [5], who reported a value of 9.1 ± 6.9 ml/min/kg in six male SD rats, and to the mean value of 10.3 ml/min/kg found by Grundy et al. [6]. Several other reports give values ranging from about 2.4 to 15.5 ml/min/kg [4,7-9]. Thus, although the reported systemic clearance of nifedipine in the rat is variable, the clearance of the drug is low relative to hepatic blood flow Q_h values as reported in the literature for this animal (55 to 80 ml/min/kg) [7,8].

Although the effect of malnutrition on drug metabolism is conflicting in literature reports, there are some results that are comparable to these found in the present study. Manjgaladze et al. [9] reported a 40-70% decrease in cytochrome P450 2C11 (CYP2C11) expression in male rats when they were subjected to food restriction. Buchanan et al. [10] studied antipyrine pharmacokinetics in kwashiorkor children and found that antipyrine half-life was longer in the malnourished than in the healthy individuals. Narang et al. [11]

and Homeida et al. [12] performed similar studies and found that antipyrine half-life was longer in malnourished humans than that reported for healthy individuals.

Nifedipine is metabolized by oxidative mechanisms, involving P-450_{UT-A} and P-450_{PCN-E} in the rat [2] [these isozymes have now been renamed as cytochrome P450 (CYP) 2C11 and CYP3A, respectively] to a pharmacologically inactive nitropyridine analogue, which is subsequently metabolized to more polar compounds. Over 80% of the primary metabolic reaction to form the nitropyridine analogue is catalyzed by CYP2C11 [1]. In human beings nifedipine is oxidized solely by CYP3A4 [13]. In an immunoblotting analysis conducted by Guengerich and his colleagues [2], it was found that more than 90% of the nifedipine oxidase activity in human liver microsomes was inhibited by anti-$P450_{NF}$. The inhibited isozyme is now called CYP3A4. Rat CYP2C11 shares most of the substrates with human CYP3A4 [13], and nifedipine is a typical example. From the present study, it is apparent that total body clearance of nifedipine was diminished in malnourished rats, and it can be interpreted that the activity of CYP2C11 in malnourished rats was decreased. Since malnutrition impairs the activities of CYP2C11 in rats, and if this observation can be extrapolated to humans, it is likely that malnutrition may impair the activity of the most abundant human isoenzyme CYP3A4.

Nifedipine was found to have a relatively high volume of distribution at steady state (V_{ss}, Table 4.2). This indicates that, despite high plasma protein binding [4], the drug is highly bound to extravascular tissues in rat. Malnutrition decreased the volume of distribution of nifedipine from about 320 ml/kg to 270 ml/kg. The decrease of volume of

distribution in PCM rats could be the result of the diminished protein binding in the tissue and the consequence of diminished lipid composition in the tissue compartment. Although the concentrations of serum albumin and total serum protein were not significantly different in the malnutrition group and the control group, (suggesting that the plasma protein binding might not change much), the protein binding in extravascular tissues may have changed. Nifedipine is a lipid soluble drug and practically insoluble in water, with a saturated solubility of approximately 10 μg/ml at 37°C. For malnourished subjects, lipid composition in the tissue is diminished; therefore, the amount of nifedipine staying in the tissue is decreased.

4.4 CONCLUSIONS

Total body clearance of nifedipine is diminished in PCM rats; and the values of AUC and $t_{1/2}$ in malnourished rats are much higher than those in control animals. V_{ss} is also significantly changed in malnourished rats. From the results of this *in vivo* study, it is evident that malnutrition impairs CYP2C11 activity in malnourished adult rats. Since CYP2C11 in rat and CYP3A4 in human share many substrates for metabolism, it is postulated that PCM may have a significant effect in altering drug metabolism involving the most abundant CYP isoenzyme in humans.

The rate of metabolism of many drugs is an important and critical factor affecting the intensity and duration of drug action. Diet and nutritional status are two environmental variables that account for a substantial proportion of variation in drug

metabolism. Data from this study suggest that for malnourished patients, dosage adjustment may be required and therapeutic monitoring may be necessary.

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Table 4.1 Mean (± SD) physical parameters in malnourished adult rats after two months of food restriction (n = 6/group)

Parameters	Malnourished	Control
	Average	Average
Body weight (g)	270 ± 12*	450 ± 29
Serum albumin (g/L)	35.0 ± 4.2	36.5 ± 3.1
Total serum protein (g/L)	67.5 ± 9.1	68.9 ± 7.6

*$P < 0.05$ compared with controls

Table 4.2 Comparison of pharmacokinetic parameters of nifedipine (6mg/kg) *iv* in control and malnourished adult rats (n=6/group).

Parameters	Malnourished	Control
$AUC_{0\text{-last}}$ (μg min/ml)	1194 ± 178 *	544 ± 80
$AUC_{0\text{-}\infty}$ ((μg min/ml)	1206 ± 188 *	581 ± 82
$t_{1/2}$ (min)	43 ± 7 *	21 ± 4
CL_{TB} (ml/min/kg)	4.5 ± 0.7 *	10.8 ± 1.3
V_{ss} (ml/kg)	270 ± 10 *	320 ± 21

$AUC_{0\text{-last}}$: Area under the drug concentration-time from 0 to last sampling time.

$AUC_{0\text{-}\infty}$: Area under drug concentration-time from 0 to infinity.

CL_{TB}: total body clearance. $t_{½}$: half-life. V_{ss}: volume of distribution at steady state.

*$p < 0.05$ compared with controls

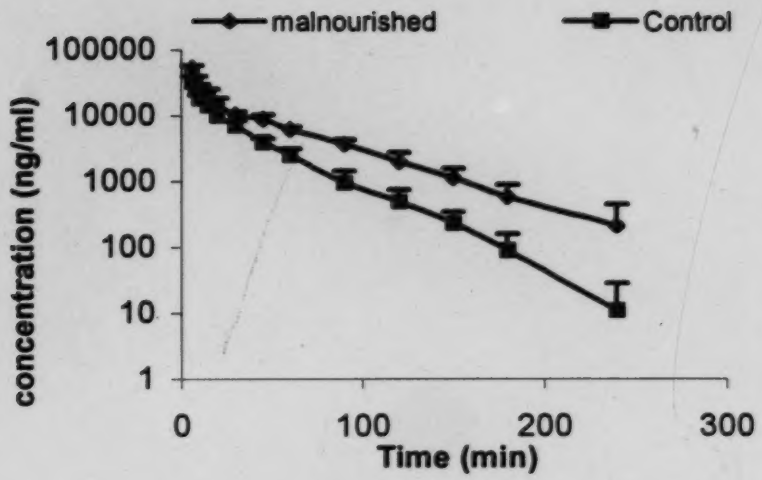

Figure 4.1 Single dose nifedipine (6mg/kg) plasma concentration-time profile in male SD rats after intravenous infusion for 4 minutes. Data are presented as arithmetic mean values ± SD (n=6/group).

CHAPTER 5

EFFECT OF PARENTERAL NUTRITION (PN) ON DRUG METABOLISM IN PCM ADULT RATS (*IN VITRO*)

5.1 INTRODUCTION

There are a few studies reported in the literature investigating the effect of parenteral nutrition on drug metabolism [1-4]. Most studies were conducted in normal subjects. Clinically, PN is only prescribed to malnourished patients. The effect of PN on drug metabolism in protein-calorie malnourished subjects is not well understood. The objectives of this study were to: 1. Determine the effect of PN on liver metabolic protein content and total P450 in PCM adult rats; 2. Monitor the alterations in capacity and affinity of metabolic enzymes in PN-treated PCM rats to characterize the PN effect on drug metabolism employing testosterone as a probe drug.

5.2 MATERIALS AND METHODS

5.2.1 Chemicals and Reagents

Testosterone, 6β-hydroxytestosterone, 16β-hydroxytestosterone, 2α-hydroxytestosterone, 7α-hydroxytestosterone, 16α-hydroxytestosterone and cortexolone were purchased from Steraloids Inc. (Newport, Rhode Island 02840, U.S.A.). Metofane was from Janssen (North York, Ontario). All ingredients of the potassium phosphate

buffer (BDH Inc. Toronto, Ontario, Canada) were ACS grade. Glycerol and a reduced form of NADPH and disodium ethylenediaminetetraacetate (EDTA) were purchased from Sigma Chemical Co. (St. Louis MO USA). Bio-Rad reagent was supplied by Bio-Rad Laboratories (Richmond, CA, USA). Sodium heparin solution 1,000 i.u./ml was supplied by Leo Pharma Inc. (Ajax, Ontario, Canada) and saline was supplied by Baxter Co. (Toronto, Ontario, Canada).

5.2.2 Jugular Vein Cannulation for PN study

Male Sprague-Dawley (SD) rats, after two months of diet control (see chapter 3 for details), weighing 245-275 g for malnourished rats (12 rats per group) and 410- 440 g for control ones (12 rats per group), were fasted overnight then weighed and anesthetized with metofane (Pitman-Moore Ltd., Mississauga, Ontario, Canada). A small longitudinal incision was made in the skin of each rat over the right jugular vein, which was then made accessible by clearing the surrounding tissues. The vein was catheterized with a 3 cm silastic tubing (O.D. 0.047" and I.D. 0.025", Dow Corning Corp., Midland, MI USA). The catheter was then tunneled subcutaneously and exteriorized in the scapular region where it was connected to a coiled metallic spring mounted on a swivel (Rodent Single Channel Swivel, Alice King Chatham Medicla Arts, Los Angeles, CA) [5]. This connection permitted free mobility of the subject. Surgical equipment was sterilized prior to each surgery and the entire surgical procedure was carried out aseptically in a laminar flow hood. After each surgery, the animal was placed in a metabolic cage, which was housed in a well-ventilated room where a 12 h light and dark cycle was maintained. Rats

were allowed to recover for two days before PN infusion. The 12 control rats were randomly divided into two groups. The saline control group (n=6) received 20 g food per day (Agway prolab-Formula RMH1000) and free access to water and 3 ml/hr saline infusion. The PN group (n=6) received PN infusion 3 ml/hr with free access to water but no food. In the mean time, the 12 malnourished rats were also randomly divided into two groups: the saline control and the PN group. All animals received the same treatments as that described for the healthy rats, except the saline control rats received 12 g of Agway. The health conditions and daily activity of the rats were observed closely. After eight days of PN and saline treatment, rats were sacrificed for liver microsome sample preparation.

5.2.3 PN Composition and Administration of PN Solution

The composition of the PN solution was according to that reported by Ke et al. [6]. Each liter of PN contained 242 g dextrose, 52 g amino acids (10% Travasol blend B with electrolytes, Baxter-Travenol Lab., Malton, Canada), 2 ml of multivitamin (MVI 1,000, Aqueous Multivitamin IV infusion, USV Canada Inc.) and 2.25 mmol of calcium gluconate (10% calcium gluconate injection, Squibb Canada Inc., Montreal, Canada). Solutions were prepared aseptically. All infusions were delivered by a volumetric infusion pump (model 927, IMED Corporation, San Diego, CA). The rate of infusion in all animals was 3 ml/hr.

5.2.4 Preparation of Hepatic Microsomes

After eight days of parenteral nutrition, rats were anesthetized with metofane, and then the abdominal cavity was opened for portal vein cannulation. After portal vein cannulation and the livers were perfused *in situ* with ice-cold isotonic potassium chloride solution (1.15% w/v) for about 3 min with a speed of 30 ml/min until the liver turned yellowish (color of liver when blood was cleared). The liver was excised and weighed. The following steps were performed at 4°C. The livers were finely minced and homogenized in three volumes of ice-cold isotonic potassium chloride solution (1.15% w/v) using a glass homogenizer equipped with a glass pestle (Glas-Col, Cole-Parmer instrument Co., Terre Hautein, IN, USA). The homogenate was centrifuged at 10,000 g for 30 min using a Model IEC B-20A centrifuge equipped with a No. 870 rotor (International Equipment Company, Boston, and MA, USA). The supernatant was evenly distributed into polycarbonate tubes (Ultratube, 13 × 64 mm, Nalge Company, Rochester, NY, USA) and was centrifuged at 105,000 g for 60 min in a model L-55 ultracentrifuge (Beckman Instruments Inc., Palo Alto, CA, USA, rotor type 503 Ti). The microsomal pellet was washed and re-suspended in 100 mM potassium phosphate buffer (pH 7.4). After re-centrifugation at 105,000 g for 60 min, the microsomal pellet was re-suspended in a volume of 100 mM potassium phosphate buffer, which contained 1 mM EDTA and 20% v/v glycerol (Sigma Chemical Co., St. Louis, MO, USA). The final volume was equivalent to original liver weight v/w (1 g of liver to make 1 ml microsome) and stored at -80°C in 1 ml aliquots.

5.2.5 Determination of Microsomal Protein Content

Microsomal protein was quantified using the Bradford method [7]. A standard curve was prepared using bovine plasma gamma globulin (Sigma Diagnostic, St. Louis, Mo, and USA). Bovine plasma gamma globulin was reconstituted with 40ml double distilled water to obtain a solution of 705 μg/ml. Aliquots of the stock solution (0, 2, 4, 6, 8, 10 and 12 μl) were added in triplicate to the wells of a microtitre plate (Corning Laboratory Sciences Co., Richmond Hill, Ontario, Canada). An appropriate amount of Bio-Rad reagent (Bio-Rad Laboratories, Richmond, CA, U.S.A.) was added so that the final volume in each well was 200 μl. The original sample taken was 40 μl to determine the protein concentration of liver microsomes, then diluted to a concentration by a factor of 25 to 500. A 10 μl aliquot of each diluted sample was mixed with 190 μl of Bio-Rad reagent (n = 3). Absorbance readings were taken at 590 nm using a Maxline microtitre plate reader (Fisher Scientific, Nepean, Ontario, Canada).

5.2.6 Total P450 Measurement

The method described by Omura and Sato [8] was used to measure total P450. Microsomes suspended in 0.1 M phosphate buffer, pH 7.4 (2 mg/ml of microsomal protein) were placed in two 1-cm square cells. One was for sample measurement and another was for reference. Both cells were carefully bubbled with CO at a rate of one bubble per second for one minute, then the reference cell was covered and to the sample cell was added a few milligrams of sodium dithionite. Subsequently, the sample cell was

covered. The difference in spectra of reference and sample was measured after two minutes. The concentration of total P450 was calculated using Beer's Law with an extinction coefficient of 91 $mM^{-1}cm^{-1}$.

5.2.7 Microsomal Incubation and Extraction

A final volume of 500 µl microsomal reaction mixture containing a 10 µl aliquot of a testosterone stock solution (substrate concentrations: 5, 10, 20, 40, 80, 125, 250, 500, 1000, 2500 µM), 5 mM $MgCl_2$, 5 µM $MnCl_2$, and 1 mM NADPH in 100 mM potassium phosphate buffer (pH7.4) was pre-incubated at 37°C for 5 min. The reaction was initiated by adding microsomal protein (1mg/ml) to the mixture and carried out in air in microcentrifuge tubes (Brinkmann Instruments Inc., Westbury, NY, U.S.A) at 37°C. The reaction was terminated after 15 min of incubation by adding the incubation mixture to 6 ml of methylene chloride, then 1 nmol of cortexolone (in 100 µl methanol) was added as an internal standard. The sample was vortexed on a vortex shaker (IKA-VIBRAX-VXR, Terrochem, setting at 1200) for 15 min and centrifuged (1000 *g*) for 10 min, and the methylene chloride layer was removed and dried in a speed vacuum evaporator (Speedvac SC100, Franklin electric USA). The residues were reconstituted in 80 µl of acetonitrile and 50 µl of water. A 100 µl of reconstituted solution was injected into the HPLC.

5.2.8 HPLC Assay for Testosterone

The HPLC system (Shimadzu, Tokyo, Japan) was equipped with a LC-600 pump, a SIL-9A auto injector, a SPD -6AV UV detector set at 247 nm and an IBM - compatible PC computer system equipped with the Baseline software program (Waters, Milford, MA, USA). The column used was LiChrospher 60 RP-select B C_8 5 μm id, 125× 4 mm (Merck, Darmstadt, F. R., Germany).

The separation system consisted of mobile phase A (23% of acetonitrile and 77% water) and mobile phase B (100% of acetonitrile). A gradient system started with 100% of A. Between 8-18 min, mobile phase B was programmed to increase linearly from 0 to 100%; the proportion of B was reduced linearly to 0 from 18-22 min, and this condition was held for two minutes for equilibration. The flow rate used was 2 ml/minute.

5.2.9 Statistical Analysis

All the data obtained were analyzed by the 2 × 2 factorial design with nutritional status as factor A and nutritional administration route as factor B. Two-way analysis of variance (ANOVA) was used to detect differences between groups. A value of $P < 0.05$ was considered statistically significant and the power of the test is set at 80%. It is discovered that using 6 animals in each study group, the power of the test as calculated uing the equation $n = (z\alpha + z\beta)^2 * 2\sigma^2 / \delta^2)$ is higher than 90% in all cases. Data are expressed as mean ± SD, unless stated otherwise.

5.3 RESULTS AND DISCUSSION

Table 5.1 summarizes the physical and microsomal parameters in control and malnourished adult rats after eight days of either PN or saline treatment. The control rats treated with saline or PN served as corresponding controls for the malnourished rats. For the malnourished group, the saline-treated rats had a slight reduction of body weight after surgery and eight days of saline treatment. This could have been associated with the cannulation surgery. On the other hand, the body weight of the PN-treated malnourished rats increased, suggesting PN did improve the nutritional status of these animals. For the control groups, body weight before and after saline or PN treatment did not change significantly. This indicates that the isocaloric maintenance regimens, given either by enteral (through gastrointestine tract) or parenteral routes, are equivalent.

In malnourished rats, the liver weight after PN treatment was similar to that observed in the corresponding control group ($p>0.05$). The liver weight of the malnourished rats treated with saline was less than half of that of the PN-treated malnourished rats ($p<0.05$). This could be partially explained by fat accumulation which occurred during PN treatment. During the experiment, it was observed that the texture of the livers from PN-treated rats was looser than that from the saline group. When the livers were homogenized, livers from the PN-treated rats were found to have a lot of fat sticking on the pestle.

Microsomal protein content per gram of liver was found to be the lowest in PN-treated malnourished rats, followed by saline-treated malnourished rats, PN-treated controls, and saline-treated control rats. According to our observations, malnutrition decreased microsomal protein content, which is consistent with our previous finding (see chapter 3), and these results agree with those reported in the literature [9,10]. Waterlow [10] found that malnutrition significantly impaired the rate of tissue protein synthesis and turnover. It is clear from the present data that PN significantly reduced liver microsomal protein contents (Table 5.1). This result was comparable to those documented by Lanza-Jacoby et al. [9] in that protein concentration in blood and interstitial spaces decreased with a PN. In the malnourished group of rats, PN appeared to provide more nutrients than does the restricted diet to support the body weight and liver weight gain; however, the protein content per gram of liver was diminished in PN-treated malnourished rats.

It is hypothesized that rat growth hormone (rGH) played a key role in the decrease of microsomal protein content in PCM rats since rGH is an important regulator of protein synthesis. Growth hormone and the pituitary-thyroid axis are sensitive to protein-calorie deficit [11-13]. Armario et al. [14] reported that chronic food restriction resulted in a marked decrease in serum GH, pituitary and adrenal weights, and a suppression of the pulsatile GH release. In this study, we did not observe a significant change in rGH in malnourished rats (see chapter 7 for rGH results). So the mechanism of alteration of microsomal protein in PCM rats is still unknown. In PN subjects, the major reason for the decrease in microsomal protein was postulated to be due to hepatic complications such as

steatosis, cholestasis, or hepatocyte necrosis. According to literature [15-17], these hepatobiliary complications are common in adult subjects receiving PN. With progressive hepatobiliary dysfunction and liver damage from PN, basal hepatic function was impaired, and this resulted in decreased protein concentration in intravascular and interstitial spaces since the protein synthesis and export were impaired [9].

The total P450 in liver microsomes in the four groups were found to be significantly different. Parallel to that observed with microsome protein levels, total P450 levels in PN-treated malnourished rats were the lowest, and these values were even lower than those of the corresponding saline-treated animals. Total P450 levels in saline-treated malnourished rats were lower than those of the saline-treated control animals, while total P450 levels in PN-treated control rats were also lower than those in the saline control animals. The present results confirm our previous observations (refer to chapter 3) which showed that malnutrition decreased the total P450 content. Our data on PN treatments agree with the results reported by Knodell and coworkers [18]. They found that 7 days continuous *iv* administration of PN *via* the jugular vein in SD male rats produced a pronounced reduction in microsomal P450 concentration. An *in vitro* study conducted by Raftogianis et al. [2] in rat liver microsomes after PN treatment for more than 10 days showed a 47% reduction in P450 content.

Figure 5.1 shows 16α-hydroxylation of testosterone has a large capacity, since it becomes saturable at very high substrate concentration (>2.0 mM). Lineweaver-Burk

plots are linear (Figure 5.2) implying that no substrate or metabolite inhibition or induction occurs.

Similar to the PCM *in vitro* study, Table 5.2 indicates that the activities of CYP2C11, CYP3A, and CYP2A1 in saline-treated malnourished rats were significantly lower than those in saline-treated controls. For the PN-treated groups, the activities of CYP2C11, and CYP3A were significantly impaired compared to those in the saline counterparts, while the activities of CYP2B1 and CYP2A1 were not significantly changed. These results are consistent with literature reports. Knodell and coworkers [18] reported that continuous PN *via* the jugular vein produces selective effects on hepatic cytochrome P450. They observed that continuous PN significantly reduced two hepatic constitutive forms of P450, CYP3A and 2C11, compared with control animals receiving the same diet by enteral route. In contrast, the amounts of hepatic cytochrome P450 2A1 and 2C6 were unchanged.

Most nutrients participate directly or indirectly in the functioning of enzyme systems involved in the biotransformation of drugs, such as competing with enzymes for metabolic and protein binding sites, or inducing or inhibiting CYP enzymes [19]. Cruciferous vegetables, for instance, induce CYP1A2 and grapefruit juice inhibits CYP3A4. High concentrations of circulating nutrients in the blood stream during PN may change the composition and activity of drug metabolism enzmyes by inducing and /or inhibiting isoenzymes differently as the nutrients such as carbohydrate or vitamins involve different isoenzymes for metabolism. Altered gene transcription due to an absence or

reduced concentrations of gastrointestinal hormones in the portal blood elicited by intravenous feeding is also a possible explanation for the selected alteration of the isoenzymes [18].

What was unexpected in the present study was that the microsomal protein, total P450, capacity and affinity of CYP2C11 and CYP3A were further impaired in PN-treated malnourished rats. Conventionally, it is assumed that PN restores the nutrition status (which is true in terms of body weight and liver weight according to our results); consequently, it would be effected to restore microsomal protein and total P450 production and the activity of the enzymes in PCM subjects. However, the results showed us that PN did not restore the enzymes' activities. Instead it further impaired capacities and affinities of some enzyme, including some major isoenzymes such as CYP2C11 and CYP3A in rats. As documented in the literature [20-22], cytokines down-regulate drug transformation. A malnourished subject could already be immune impaired and cytokine levels could have been already elevated; hence the subject could be more fragile to any further immune insult such as PN [23]. Studies conducted by Chandra and his co-workers [24-26] showed PCM was associated with a significant impairment of cell-mediated immunity, phagocyte function, complement system, and secretory immunoglobulin A antibody concentrations. The lymphoid tissues, particularly the thymus, were found to be atrophied. There was a reduction in delayed cutaneous hypersensitivity, fewer T cells, especially T helper cells, decreased thymullin activity, impaired secretory immunoglobulin A antibody response, decreased affinity, and reduced concentration and activity of

complement components and phagocytes [26,27]. With the already impaired immune system, plus another immune insult such as PN that adversely affects immune function [28], the immune system could further deteriorate, and metabolism could be further decreased, further impairing activities of CYP2C11 and CYP3A. Similar to the results in PN control rats, CYP2B1 and CYP2A1 activities did not show a significant change in PN malnourished rats when compared to those of the malnourished rats with enteral food.

5.4 CONCLUSIONS

From this study, it is concluded that PN diminished microsomal protein levels and total P450 in healthy rats. PN impaired the capacity (V_{max}) and affinity (K_m) of the 16α-, and 2α-testosterone hydroxylation catalyzed by CYP2C11 and of 6β-testosterone hydroxylation that is mediated by CYP3A. However, the capacity and affinity of 16β-hydroxylation of testosterone catalyzed by CYP2B1 and catalysis 7α-hydroxytestosterone formation *via* CYP2A1 did not change significantly between the PN group and the enteral nutrition group. Although PN restored the body weight and liver weight of malnourished rats, it further decreased the microsomal protein, total P450, and capacity and affinity of CYP2C11 and CYP3A in malnourished adult rats.

Based on the results observed in this study, it is clear that, although PN can rehabilitate the nutritional status of PCM rats, the animals' drug metabolizing abilities are even worse than those of the malnourished animals with enteral food. The study

suggested that, if this observation could be extended to humans, the use of drugs in patients receiving PN who are malnourished, should be done with caution.

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Table 5.1 Mean (± SD) physical and microsomal parameters in malnourished adult rats after eight days of parenteral nutrition and saline (n = 6/group)

Parameters	PN–PCM	S–PCM	PN–C	S–C
Body weight before PN (g)	262 ± 17	267 ± 16[b]	433 ± 27	421 ± 24
Body weight after PN (g).	361 ± 15[a]	261 ± 15[b]	434 ± 26	423 ± 31
Liver weight (g)	15.1 ± 1.8[a]	7.1 ± 0.8[b]	16.1 ± 2.7	16.3 ± 1.6
Prot./liver(mg/g)	24.4 ± 2.4[a]	30.0 ± 2.1[b]	33.6 ± 2.0[c]	40.6 ± 2.7
P450/ liver (nmol/g)	12.5 ± 1.8[a]	16.5 ± 1.2[b]	18.1 ± 1.8[c]	23.8 ± 1.4

[a b c] $P<0.05$

PN: Parenteral Nutrition PCM: Protein Calorie Malnourished

S: Saline C: Control

[a] Parameters in PN malnourished group are significantly different from those in saline malnourished group ($P<0.05$),

[b] Parameters in saline malnourished group are significantly different from those in saline control group ($P<0.05$),

[c] Parameters in PN control group are significantly different from those in saline control group ($P<0.05$).

Table 5.2 Mean (± SD) kinetic parameters of testosterone metabolism in control and malnourished adult rat liver microsomes after eight days of parenteral nutrition and saline (n = 6/group)

	V_{max} (pmol/min/mg)				K_m (μM)			
Pathways	**PN-PCM**	**S-PCM**	**PN-C**	**S-C**	**PN-M**	**S-M**	**PN-C**	**S-C**
16β-hydroxylation	46.1 ± 5.3	48.3 ± 6.3	80.0 ± 10.2	86.2 ± 11.1	39.0 ± 4.4	38.1 ± 4.9	37.9 ± 4.0	36.9 ± 4.1
2α-hydroxylation	1123 ± 111[a]	2702 ± 211[b]	3448 ± 351[c]	4445 ± 451	29.2 ± 3.4[a]	22.6 ± 2.9[b]	24.1 ± 2.6[c]	15.6 ± 2.0
16α-hydroxylation	970 ± 101[a]	1851 ± 191[b]	2010± 211[c]	2564 ± 261	30.2 ± 3.7[a]	19.1 ± 3.1[b]	17.8 ± 2.2[c]	12.8 ± 2.4
7α-hydroxylation	196 ± 21	205 ± 225[b]	294 ± 30	303 ± 31	14.6 ± 2.1	13.1 ± 2.1	12.9 ± 2.0	11.3 ± 1.4
6β-hydroxylation	208 ± 21[a]	357 ± 36[b]	416 ± 42[c]	667 ± 70	32.2 ± 4.0[a]	21.5 ± 3.1[b]	21.5 ± 2.8[c]	15.4 ± 2.6

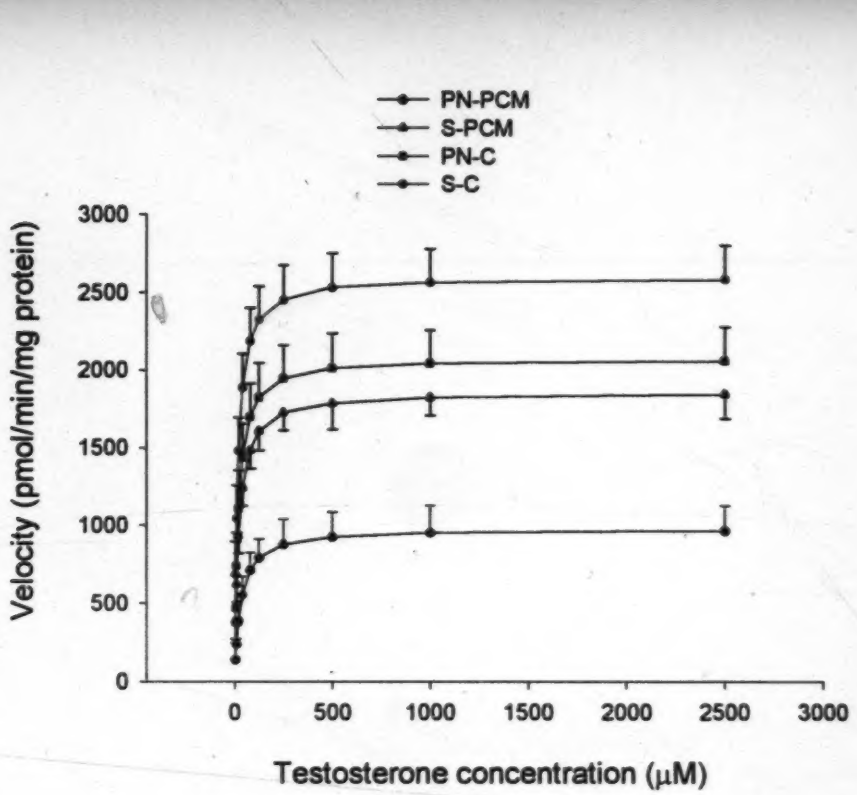
PN: Parenteral Nutrition PCM: Malnourished

S: Saline C: Control

[a] Parameters in PN malnourished group are significantly different from those in saline in malnourished group ($P<0.05$),

[b] Parameters in saline malnourished group are significantly different from those in saline in control group ($P<0.05$),

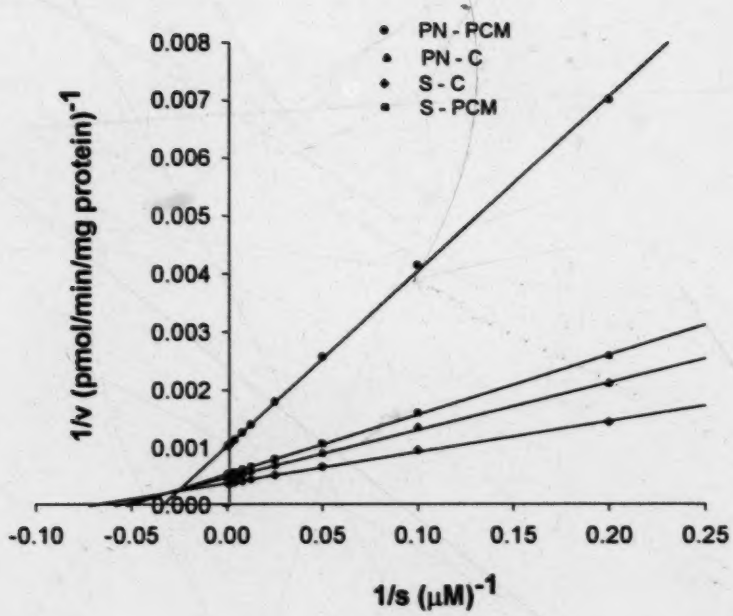
[c] Parameters in PN in control group are significantly different from those in saline in control group ($P<0.05$).



PN: Parenteral Nutrition PCM: Malnourished

S: Saline C: Control

Figure 5.1 Michaelis-Menten plots of 16α-hydroxytestosterone formation in PN-treated control and PCM adult rat liver microsomes. Substrate concentration ranged from 5-2500 μM. Results are expressed as mean ± S.D.



PN: Parenteral Nutrition PCM: Malnourished

S: Saline C: Control

Figure 5.2. Lineweaver-Burk plot of 16α-hydroxytestosterone formation in PN-treated control and PCM adult rat liver microsomes. Substrate concentration ranged from 5-2500 μM

CHAPTER 6

EFFECT OF PARENTERAL NUTRITION (PN) ON DRUG METABOLISM IN PCM ADULT RATS (*IN VIVO*)

6.1 INTRODUCTION

Most drug metabolism studies involving parenteral nutrition (PN) have been conducted in normal subjects [1-4], but the real clinical situation is that, when a patient needs PN, most likely s/he is already malnourished. However, the effect of PN on drug metabolism in protein-calorie malnourished (PCM) subjects is not well characterized in the literature. *In vitro* and *in vivo* studies (chapters 3 & 4) showed that PCM impaired drug metabolic enzyme activity. PN has been shown to further impair drug metabolism including that catalyzed by CYP2C11 activity in PCM rats *in vitro* (chapter 5). CYP2C11 catalyzes 80% of nifedipine metabolism in the Sprague-Dawley (SD) male rat [5]. Rat CYP2C11, a major isoenzyme in rats [6] and CYP3A4, a major isoenzyme in humans [7] share most of substrates for metabolism [7]. By employing nifedipine as a probe drug, the objective of this study is to elucidate the effect of PN on nifedipine clearance in PCM rats through an *in vivo* approach, so that the PN effect on drug metabolism in PCM subjects will be revealed.

6.2 MATERIALS AND METHODS

6.2.1 Chemicals and Reagents

Nifedipine and nitrendipine were purchased from Sigma Inc. (Mississauga, ON, Canada). Acetonitrile and methanol were HPLC grade and purchased from Fisher Scientific (Nepean, Ontario, Canada). Methylene chloride and isooctane were HPLC grade and purchased from BDH (BDH Inc., Toronto, Ontario, Canada). Acetic acid, methyl-t-butyl ether (MTBE) and triethylamine (TEA) were analytical grade and were purchased from Fisher Scientific (Nepean, Ontario Canada). Polyethylene glycol (PEG 400) was obtained from Union Carbide Chemicals (Danbury, CT, USA).

6.2.2 Jugular Vein Cannulation, PN Composition and Administration

Each male SD rat after malnutrition treatment (refer to chapter 3, section 1.2.2 for details) weighing 245-275 g for malnourished rats and 410-440 g for control ones was weighed and anesthetized with Metofane (Pitman-Moore Ltd., Mississauga, Ontario, Canada). A small longitudinal incision was made in the skin of each rat over the right jugular vein, which was then made accessible by clearing the surrounding tissues. The vein was catheterized with 3 cm silastic tubing (O.D. 0.047" and I.D. 0.025", Dow Corning Corp., Midland, MI USA). The catheter was then tunneled subcutaneously and exteriorized in the scapular region where it was connected to a coiled metallic spring mounted on a swivel (Rodent Single Channel Swivel, Alice King Chatham Medicla Arts,

Los Angeles, CA) [8]. This connection permitted free mobility of the subject. Surgical equipment was sterilized prior to each surgery and the entire surgical procedure was carried out aseptically in a laminar flow hood. After each surgery, the animal was placed in a metabolic cage, which was housed in a well-ventilated room where a 12 hr light and dark cycle was maintained. Rats were allowed to recover for two days before infusion, and during this time they were given nutritionally balanced diet (Agway prolab-Formula RMH1000) (malnourished rats 12 g/day, control rats 20 g/day) and free access to water.

The composition of the PN solution was according to that reported by Ke et al. [9]. Each liter of PN contained 242 g dextrose, 52 g amino acids (10% Travasol blend B with electrolytes, Baxter-Travenol Lab., Malton, Canada), 2 ml of multivitamin (MVI 1,000, Aqueous Multivitamin *iv* infusion, USV Canada Inc.) and 2.25 mmol of calcium gluconate (10% calcium gluconate injection, Squibb Canada Inc., Montreal, Canada). Solutions were prepared aseptically. All infusions were delivered by a volumetric infusion pump (model 927, IMED Corporation, San Diego, CA). The rate of infusion in all animals was 3 ml/hr.

Two days after surgery, the rats were divided into four groups, twelve control rats were randomly divided into two groups, one group of 6 rats (S-C group) was the saline control group which received 20 g food (Agway prolab-Formula RMH1000) per day and free access to water and 3 ml/hr saline infusion, and the other 6 rats (PN-C group) received 3 ml/hr PN infusion, had free access to water and no access to food. The 12 malnourished rats were also randomly divided into two groups: 6 rats (S-PCM group)

were the saline control animals and the other 6 received PN (PN-PCM group). The treatment procedures for these two groups of rats were identical to that described for the healthy rats except that the saline control ones received 12 g of the same food. General health conditions and daily activity were observed and recorded daily.

Eight days after either saline or PN treatments, rats were given nifedipine 6 mg/kg (a 4-minute infusion *via* the jugular vein).

6.2.3 Dosage Form Preparation

Nifedipine solutions were prepared by adding nifedipine powder to PEG 400 (5mg/ml) the day before administration to allow sufficient time for drug solubilization. To protect from light, the container holding the drug solution was wrapped with aluminum foil and stored at room temperature. To prevent nifedipine degradation, all dosage solutions were prepared and handled in a room illuminated with sodium light.

6.2.4 Nifedipine Administration and Blood Sampling

Nifedipine (5 mg/ml PEG 400) was given by a 4-minute *iv* infusion. Before nifedipine administration, about 500 μl blood was taken out for washing the catheter. The dose was injected slowly from 0 to 4 min through the jugular vein cannula. Blood samples of approximately 150–250 μl were drawn through the jugular vein cannula with heparinized-coated 1-ml tuberculin syringes at time 0, 6, 8, 10, 15, 20, 30, 45, 60, 90, 120, 150, 180 and 240 min and the same volume of saline was injected to replace the lost

blood. Immediately after blood sampling the cannula was rinsed first with about 40 µl blood, and then twice with about 0.1 ml saline (the same size as the blood sample taken). Each rinse was carried out very slowly to make sure all nifedipine on the tube was washed out (This procedure is necessary to prevent any carryover from drug administration). Each blood sample was centrifuged for 5 min at 2500 g. Plasma was harvested and transferred to tubes that were wrapped with aluminum foil. These samples were stored at -20^0C until analyzed.

6.2.5 Analysis of Plasma Nifedipine

Plasma nifedipine concentrations were measured by the HPLC method reported by Grundy et al. [10] with some modifications. The internal standard used was nitrendipine. The analytical method is briefly described as follows: a 100 µl of plasma sample were basified with 1.0 M sodium hydroxide, extracted with methyl-t-butylether (MTBE):iso-octane (75:25, v/v) and subsequently the resulting organic phase was evaporated with a vacuum evaporator. The residue was reconstituted with 200 µl of mobile phase (methanol:water:acetic acid:triethylamine, 65:34:1:0.03, v/v). A 100 µl of sample was injected onto a Nova-Pak 8 × 100 mm radial pack column containing 4µm C8 as stationary phase (Waters, Mississauga, ON., Canada). Nifedipine was detected using a SPD–6A UV-Vis detector (Shimadzu Corporation, Tokyo, Japan) set at 250 nm. Calibration curves were linear (r^2 >0.99) from 10 to 2500 ng/ml. All HPLC runs had a total of nine quality control samples, including 3 each at low (10ng/ml), medium (200

ng/ml) and high (1000 ng/ml) concentrations. Intra- and inter-day variability was less than 7%. Accuracy and precision were within 10% over the concentration range. Baseline software (Waters, Milford, MA, USA) was used to integrate peaks and process chromatographic data.

6.2.6 Statistical Analysis

The pharmacokinetic parameters obtained were analyzed by the 2 × 2 factorial design with nutritional status as factor A and nutritional administration route as factor B. Two-way analysis of variance (ANOVA) was used to detect differences between groups. A value of $P \leq 0.05$ was considered statistically significant and the power of the test is set at 80%. It is discovered that using 6 animals in each study group, the power of the test as calculated uing the equation $n = (z\alpha + z\beta)^2 * 2\sigma^2 / \delta^2)$ is higher than 90% in all cases. Data are expressed as mean ± SD, unless stated otherwise.

6.3 RESULTS AND DISCUSSION

The mean (±SD) plasma nifedipine concentration-time profiles after *iv* dosing of nifedipine (6 mg/kg) to the four groups of animals are shown in Figure 6.1. The individual profiles obtained displayed multi-exponential decline. In a descending order, the plasma concentration was the highest in PCM rats treated with PN, followed by PCM rats treated with saline, then healthy rats treated with PN and finally healthy rats treated with saline.

Standard pharmacokinetic parameters calculated from this set of data are shown in Table 6.1. These data clearly showed that the clearance of nifedipine was impaired when the animals were suffering from malnutrition. This is reflected by the significantly higher nifedipine concentrations, lower clearance and longer half-life values obtained from the PCM animals. The data also showed that PN treatment, independent of nutritional status, significantly reduced the elimination of nifedipine in healthy and PCM animals. Interestingly, according to conventional theory, PN rehabilitates the nutritional status of malnourished subjects, therefore it may also restore the metabolic capability. However, the CL_{TB} in PN-treated malnourished rats observed in this study was even lower than that in enteral nutrition PCM rats and $AUC_{0\text{-last}}$, $AUC_{0\text{-}\infty}$, $t_{1/2}$ values were significantly higher. It means that although PN did rehabilitate the nutritional status of malnourished subjects in terms of body weight, it further impaired the elimination of nifedipine in PCM rats.

The values of V_{ss} were significantly lower in malnourished groups than those of the healthy ones. The values of V_{ss} in PN-treated healthy rats were not significantly different from the corresponding enterally fed ones. The values of V_{ss} in PN-treated PCM rats were slightly higher than those of the corresponding enterally fed ones.

The pharmacokinetic difference between the two nutritional statuses came from the effect of malnourishment, which was already observed in the malnourishment study (refer to chapter 5). The differences in CL_{TB}, $AUC_{0\text{-last}}$, $AUC_{0\text{-}\infty}$, and $t_{1/2}$ between the enteral nutrition and parenteral nutrition animals resulted from the effect of PN-the nutrition administration rout. This is comparable to findings by others. Knodell and

coworkers [11] found that 7 days of continuous *iv* administration of PN *via* the jugular vein in SD male rats resulted in an approximately 50% reduction in pentobarbital hydroxylase activity, and a 40% decrease in meperidine demethylase activity in rats, compared with the control animals fed with an identical diet by the enteral route.

PN infusion may cause an instant elevation of the concentration of circulating nutrients in the blood stream. The high concentration of nutrients in the blood may change the composition and activity of drug metabolism enzymes by inducing and/or inhibiting isoenzymes differently, and also acutely compete with drugs for the metabolic enzyme sites in the liver [12]. The major enzyme systems that metabolize drugs are also responsible for the metabolism of a variety of other exogenous and endogenous compounds, including macronutrients from PN. These nutrients may also acutely compete with drugs for the plasma protein binding sites because some drugs and nutrients share the same binding sites [12-15]. As a result of competition, nifedipine, a lipophilic drug, could be displaced from the hepatic metabolizing enzyme sites, which could result in an acutely altered drug elimination from the body during PN infusion. For the PN-treated malnourished subjects, with depressed enzyme systems resulting from malnutrition and increased nutrient levels in the blood stream, the decrease in drug disposition was more pronounced.

Results from our lab demonstrated that an increase of endotoxin and cytokine levels contributed to the impairment of drug metabolism in PN subjects. In malnourished subjects, the immune system could be impaired. Studies conducted by Chandra and co-

workers [16-18] showed that PCM was associated with a significant impairment of cell-mediated immunity, phagocyte function, complement system, secretory immunoglobulin A antibody concentrations, and cytokine production. The lymphoid tissues, particularly the thymus, were found to be atrophied. There was a reduction in delayed cutaneous hypersensitivity, fewer T cells, especially T helper cells, decreased thymullin activity, impaired secretory immunoglobulin A antibody response, decreased affinity, reduced concentration and reduced activity of complement components, and phagocyte dysfunction [19,20]. With the already impaired immune system, plus another immune insult such as PN that adversely affect immune function [21], the immune system could be further delibitated, and hence, metabolism could be further decreased.

No significant difference was observed in volume of distribution between the two nutritional administration routes in healthy groups. This is consistent with reports in the literature. Ross et al. [22] reported that the distributions of ampicillin and antipyrine were not significantly influenced by PN, and that there was no significant difference in the apparent volume of distribution between the control group and the PN group in Fischer 344 male rats. For salicylic acid, phenytoin, and valproic acid [23], their distributions are altered with the increase of free fatty acid concentrations in PN solution. This might be due to the presence of fat emulsions that alter the binding characteristics of drug to serum proteins. The volume of distribution in PN-treated PCM rats is slightly greater than that of enterally fed PCM rats, although statistical significance was not reached.

6.4 CONCLUSIONS

Parenteral nutrition impaired nifedipine elimination in healthy rats. Parenteral nutrition further impaired nifedipine total body clearance in PCM rats. The values of AUC and $t_{1/2}$ were further increased in PN-treated PCM rats, but the volume distribution did not change significantly in PN rats. It was observed that the impairment of CYP2C11 activity in PN subjects was more pronounced in PCM rats in that more than 80% of nifedipine is metabolized by CYP2C11 in male rats. If this observation can be extended to humans, the use of drugs in patients receiving PN who are malnourished has to be done with caution, especially when it involves the major isoenzyme CYP3A4, since CYP2C11 in rat and CYP3A4 in human share most substrates for metabolism [7].

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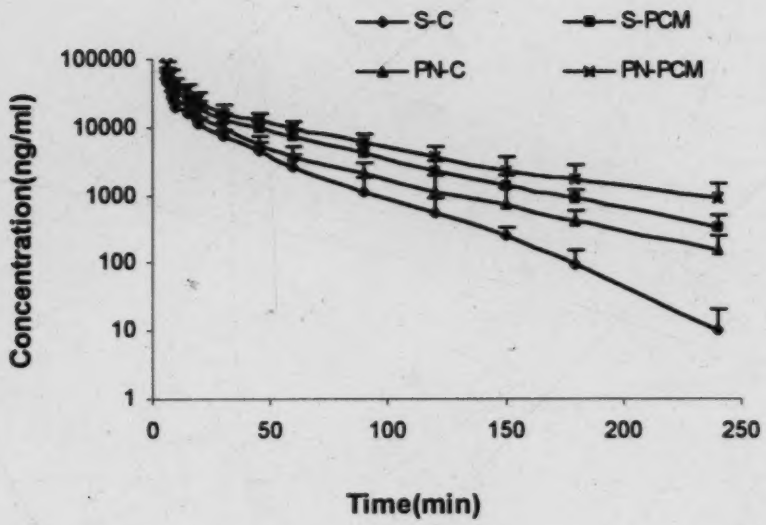
Table 6.1 Comparison of pharmacokinetic parameters of nifedipine (6mg/kg *iv*) in control and malnourished adult rats that are treated with PN or saline (n=6/group)

Parameters	PN-PCM	S-PCM	PN-C	S-C
Body weight before PN (g)	260 ± 27	267 ± 20[b]	413 ± 21	420 ± 23
Body weight after PN (g).	370 ± 17[a]	265 ± 19[b]	424 ± 20	426 ± 23
AUC $_{0\text{-last}}$ (μg min/ml)	1622 ± 170[a]	1230 ± 159[b]	789 ± 98[c]	480 ± 90
AUC $_{0\text{-}\infty}$ (μg min/ml)	1810 ± 171[a]	1340 ± 159[b]	840 ± 104[c]	523 ± 91
$t_{1/2}$ (min)	51.3 ± 5.4[a]	40.6 ± 4.4[b]	30.6 ± 3.2[c]	21.0 ± 2.9
CL_{TB} (ml/min/kg)	3.4 ± 0.5[a]	4.6 ± 0. 4[b]	7.3 ± 0.7[c]	11.5 ± 1.1
Vss (ml/kg)	270 ± 26	260 ± 39[b]	319 ± 29	345 ± 27

PN: Parenteral Nutrition PCM: Malnourished

S: Saline C: Control

- [a] Parameters in PN malnourished group are significantly different from the corresponding saline controls,
- [b] Parameters in saline malnourished group are significantly different from those in saline controls,
- [c] Parameters in PN control group are significantly different from those in saline controls
- Value = mean ± SD, *$p<0.05$



PN: Parenteral Nutrition PCM: Malnourished
S: Saline C: Control

Figure 6.1 Mean (±SD) plasma nifedipine concentration-time profiles after *iv* dosing of nifedipine (6mg/kg) to malnourished and healthy rats that received PN or saline (n=6/group). Results are expressed as mean ± S.D.

CHAPTER 7

RAT GROWTH HORMONE STUDY

7.1 INTRODUCTION

The *in vitro* and *in vivo* studies in protein-calorie malnutrition (PCM) clearly showed that rats with PCM have compromised drug metabolizing ability (chapters 3 & 5). The mechanisms that are responsible for the change, however, are not well understood. It is well known that growth hormone and the pituitary-thyroid axis are sensitive to protein-calorie deficit [1-3] and the constitutive male-specific CYP2C11 expression in rat liver is developmentally up-regulated at the transcriptional level by the male pattern of growth hormone (GH) secretion [3,4]. The objective of this study was to determine whether a change in the pattern of rGH secretion is a factor in diminishing drug metabolism in rats with PCM.

7.2 MATERIALS AND METHODS

7.2.1 Chemicals and Reagents

rGH and pituitary hormones were from UCB Bioproducts (Nanterre, France). rGH was calibrated against rGH-RP2 from the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) (Bethesda, USA). Standard chemicals were from Merck (Darmstadt, Germany) or Sigma (St-Louis, MO, USA). Normal goat serum

was from Institute Pasteur (Paris, France). Assays were conducted in 96-well microtiter plates (Immuno-NUNC Maxisorp) from Nunc (Denmark). Microtitration was conducted using a microtiter plate washer-120 and a Multiskan MCC from Titertek Flow (Helsinki, Finland).

7.2.2 Animals and Animal Care

Male Sprague-Dawley (SD) rats weighing 190-210 g were supplied by Biosciences Animal Services, University of Alberta. All animals were housed in the Dentistry-Pharmacy Building Animal Service Facility and were fed Agway (Prolab-Formula RMH1000, PMI Feeds, Inc., St. Lois, MO) before the experiments. In the first study, pediatric rats were used (190-210 g), and in the second study adult rats (340-360 g) were employed.

Pediatric rats: Male SD rats (190-210 g) were housed individually with a 12-hr light/dark cycle at 21-22°C. The animals had access to tap water and a nutritionally balanced Agway *ad libitum* during the initial 4-day acclimation period. The feed was weighed daily to determine the amount of food consumed by each rat. After the acclimation period, the animals were randomly divided into two groups (n=5 per group). Group 1, the control group, had free access to food (Agway) and water, while group 2, the experimental group, was fed 10 g of the same food per day and had free access to water. Rats were maintained on the assigned diet for 8 weeks.

Adult rats: Male SD rats (190-210 g, about 40 days old) had access to tap water and Agway *ad libitum* until the rats were 340-360 g (three months old). By that time, the rats reached adulthood. In the following experiments, similar protocols used for the pediatric rat study were employed, except that the control animals had 20 g of food (Agway) daily and free access to water to maintain the body weight, while group 2 animals were fed 12 g of the same food and had free access to water. The assigned diets were maintained for two months.

Assessment of Nutrition Status: Rats were observed for general activity and alertness daily. Serum albumin and total serum protein levels were measured every two weeks and weight measurement was taken twice a week throughout the study period.

Serum Albumin and Total Serum Protein Measurement: The tail clip method was used for blood sampling. After rubbing the tip of the tail with a cotton ball soaked with 75% of alcohol, approximately 2 mm of the tail was clipped using a sterilized razor blade. Blood sample (50 μl) was taken with a capillary tube. After that, silver nitrate sticks were used to stop the bleeding immediately. Blood samples were centrifuged for 5 min at 2,500 g to obtain serum and then the serum samples were frozen at –20°C until analyzed. Serum albumin and globulin were determined using direct dye binding with Bromocresol green, which was done by the staff at the Surgical Medical Research Institute, University of Alberta.

7.2.3 Serum Sampling for rGH Measurement

After the malnutrition treatment, rats were subjected to jugular vein cannulation. The animals were allowed to recover for two days. On the third day, blood samples were taken from jugular vein at 8:00 am, 11:00 am, 2 pm and 8 pm. Serum was harvested and immediately frozen at –20°C until rGH analysis.

7.2.4 Measurement of Rat Growth Hormone

The serum samples were sent to Paris, France (Dr. Grouselle's lab) for rGH analysis. A published method by Dr. Ezan's group was used to quantify rGH in rat serum [4]. Briefly, rGH assays were performed in enzyme immunoassay (EIA) phosphate buffer (0.1 M at pH 7.4) containing 0.15 M NaCl, 0.001 M EDTA, 0.1% BSA, 0.01% sodium azide and 0.2% Triton X-100. The 96-well microtiter plates were coated with rabbit polyclonal antibodies specific for goat IgG at a concentration of 10 μg/ml for one night at room temperature and saturated in the EIA buffer without Triton X100. Before use, plates were extensively washed with 0.01 M phosphate buffer pH 7.4 containing 0.1% Tween 20. The total volume of the assay solution was 150 μl. Plasma and culture medium samples were diluted in enzyme immunoassay buffer. Reagents were dispensed as follows: 50 μl sample or standard rGH from UCB Bioproducts calibrated against the NIDDK standard rGH-RP2), and 50 μl rGH antiserum. After a 20 hr incubation at room temperature, 50 μl enzymatic tracer (0.25 Ellman units/ml) were added before a further 20 hr incubation at room temperature. Plates were again washed and Ellman's reagent (200

μl) was distributed into each well and incubated for 3 hr. Absorbance was measured with a spectrophotometer at 414 nm. Unknown concentrations were calculated from a model standard curve. All measurements for standards and samples were made in duplicate.

7.3 RESULTS AND DISCUSSION

Table 7.1 shows the average body weight, serum albumin and total serum protein concentrations in pediatric and adult malnourished and control rats. Although the body weight in both groups changed dramatically, serum albumin and total serum protein did not show any significant difference. Table 7.2 shows the levels of rGH in pediatric malnourished and control rats. Table 7.3 summarizes the levels of rGH in adult malnourished and control rats. The reason that blood was taken at 8 am, 11 am, 2 pm and 8 pm is because, according to the literature, these are the peak and trough times for rGH secretion [5]. The observations from this study shows that the secretion of rGH at peak and trough times was not significantly different between experimental groups and controls in either pediatric or adult studies. Figure 7.1 and Figure 7.2 indicate that the secretion pattern, as well as the concentrations of rGH in serum, were very similar in PCM and control groups of pediatric and adult rats.

It is believed that pituitary function is impaired in malnutrition subjects [6,7]. The severe growth retardation seen in PCM rats is consistent with an impaired secretion of growth hormone. The initial indications are that, in contrast to the prevailing concept, serum GH levels are actually raised in PCM patients [8]. This finding has been confirmed

by many investigators [9-15] and is now almost universally accepted. Plasma GH levels are raised in kwashiorkor patients, whereas the findings in marasmus are discordant. While some researchers have reported normal or low values [16-20], others have observed raised levels of plasma GH [21] in marasmus[14]. This controversy can be resolved by considering the values in relation to the serum albumin concentration of the patients. There is generally an inverse relationship between serum albumin and serum GH levels [15,22]. In marasmus, serum albumin is generally in the normal range [20]. Lunn et al. [23] observed that serum growth hormone would only change when serum albumin fell to less than 2.5g/100ml.

In human PCM patients, the growth hormone levels returned to normal upon refeeding [24]. The prompt response of the endocrine alterations upon refeeding suggests that nutrition intake is one of the key factors in the induction and recovery of adaptations to the diet. Thus the hormonal changes may represent a secondary adaptive mechanism in an effort to maintain muscle function as close to normal as possible. When caloric intake is restored to an effective level, an opposite sequence of events occurred (GH level went down to normal).

Some researchers reported a decrease of rGH in malnourished rats. According to the report of Armario et al. [25], food restriction changed the profile of rGH secretion. Rats that had food *ad libitum* showed a rGH secretion profile that fluctuated during the day and had peak values around 11 am and 8 pm and troughs around 2 pm and 2 am. However, the profile for the food-restricted rats (65% of the control) was a flat line,

which had a significantly different pattern and concentration from that of control. Dickerman's group also made a similar observation with starved rats [7]. Nevertheless, the study carried out by Moberg et al. [26] showed that the effects of food restriction on rGH levels were slight and transient. After 3 days of food restriction, the plasma levels of rGH were markedly depressed when compared to those of the control SD male rats. After 14 days of food restriction, the daily pattern of plasma GH levels were still depressed, but showed a trend of recovery. After 6 weeks of food restriction, the profile of daily plasma GH rhythm was essentially the same as that for the *ad libitum* fed controls. The depressed levels of GH in the 3-day and 14-day groups may be the result of adapting to the new feeding regimen since various stresses have been reported to suppress GH levels [27-31].

Actually, stress *per se* alters GH secretion. Krulich and McCann [32] reported that different types of stress in which carbohydrate metabolism did not alter influenced pituitary GH content in rats. In their study, experimental animals were exposed to five different stimuli. Splenectomy evoked a transient decrease in pituitary GH which was followed by a rise in GH activity within 60 minutes of operation. Formalin injections similarly produced an elevation of pituitary GH. Ringing a doorbell in the animal's cage intermittently for 30 minutes also elevated pituitary GH. Exposure to a temperature of 3°C for 60 min produced a depletion of pituitary GH. A biphasic response was observed in fasting; at 16 hr after onset of the fast, a marked depletion of GH was observed, then the value returned to base line or even was elevated by 40 hr and declined again at 64 hr. Fasting might be considered as a substitute for stress stimulus. At the beginning of a fast,

the nutrition balance was lost, then adaptation took place, but later on adaptation failed and GH was depleted. So a variety of apparently unrelated stimuli could alter the GH content of the pituitary. It appears that GH secretion in rat is very labile and influenced by a variety of stresses, including alterations in the supply of available nutrition. The report from Brown's group [33] also showed GH changed with different stresses in animals. Aversive conditioning produced GH alteration in most animals but only during training. After animals have "learned" to avoid shock by bar-pressing, GH secretory responses no longer appear. Lever pressing, which successfully avoids shock, is the evidence of behavioral adaptation. When adaptation develops during aversive conditioning, the conditioning situation apparently becomes nonstressful, as defined by GH secretion. Once the animal adapts to the stress, GH could return to normal.

The discrepancy between our observation and those in the literature could be associated with the ages of the animal used and with the duration and degree of the stress. In Armario's study [25], about 30 day old male SD rats were used. At this age, GH secretion might be very sensitive to environmental stress, like food restriction. After 30 days of food restriction, the animals had not yet adapted to stress. In Dicherman's study [7], SD male rats were starved for 7 days. The animals had to adapt to the dramatic environmental change, and many physical parameters such as rGH changed to balance the alteration. In some dramatic or short time stress situations, animals are not able to accommodate to the aversive situation, so alteration of rGH is observed. In our study,

after two months of food restriction, rats could have already adapted to the stress and the rGH level returned to normal.

Another parameter that should be considered is serum albumin. Researchers [15,22] observed an inverse relationship of GH with serum albumin levels. They suggested that low serum albumin might act as a stimulus for the alteration of growth hormone levels. In our study, serum albumin was in the normal range, so it is not surprising that we did not see a change in rGH.

7.4 CONCLUSIONS

From this study we found that rGH secretion did not change significantly in SD male rats after two months of food restriction in both pediatric and adult rats. The quantity and the secretion pattern of rGH were not altered in PCM rats compared to those in healthy animals. The change of GH is stress related and has a relationship with serum albumin. It is obvious that the impairment of drug metabolism in PCM rats was not caused by the alteration of rGH. The mechanisms that caused the impairment of drug metabolism in malnourished subjects require further investigation.

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Table 7.1 Average body weight, serum albumin and total serum protein in pediatric and adult malnourished and control rats (n=6/group)

	Pediatric		Adult	
Parameters	**Malnourished**	**Control**	**Malnourished**	**Control**
Body weight (g)	230 ± 14*	470 ± 10	277 ± 10*	445 ± 27
Serum albumin (g/L)	33.0 ± 2.5	35.3 ± 3.9	34.0 ± 4.8	34.5 ± 2.1
Total serum protein (g/L)	63.5 ± 11.8	66.8 ± 12.6	65.5 ± 10.1	66.1 ± 8.6

Values are means ± SD *$P < 0.05$

Table 7.2 Average serum rGH levels in pediatric malnourished and control rats (n=6/group)

Time	Malnourished (ng/ml)	Control (ng/ml)
8:00AM	7.61 ± 1.33	10.53 ± 4.24
10:00 AM	10.18 ± 1.35	11.59 ± 2.19
11:00 AM	145.67 ± 32.18	168.61 ± 42.57
2:00PM	4.92 ± 1.26	4.73 ± 1.41
8:00PM	51.75 ± 9.21	52.86 ± 8.45

Values are means ± SD

Table 7.3 Average serum rGH levels in adult malnourished and control rats (n=6)

Time	Malnourished (ng/ml)	Control (ng/ml)
8:00AM	8.71 ± 2.36	9.51 ± 3.21
10:00 AM	11.18 ± 3.34	12.59 ± 2.78
11:00 AM	115.09 ± 42.01	138.69 ± 40.55
2:00PM	5.93 ± 2.21	6.70 ± 2.49
8:00PM	49.77 ± 8.29	50.81 ± 7.48

Values are means ± SD

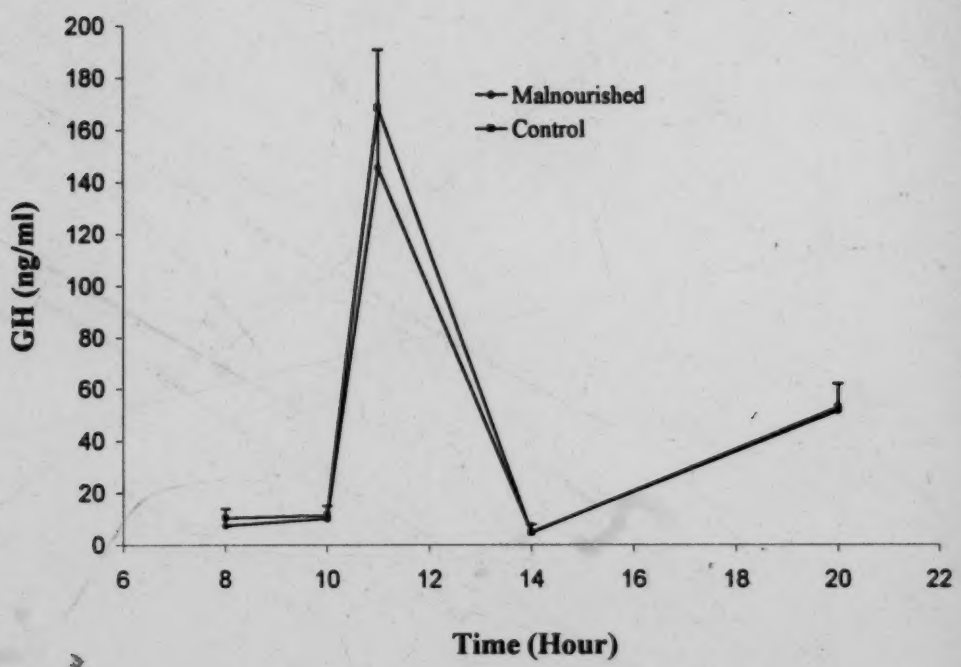

Figure 7.1 Circadian rhythmicity of rGH in pediatric control and PCM rats after two months of food restriction (n=6/group). Values are expressed as mean ± SD

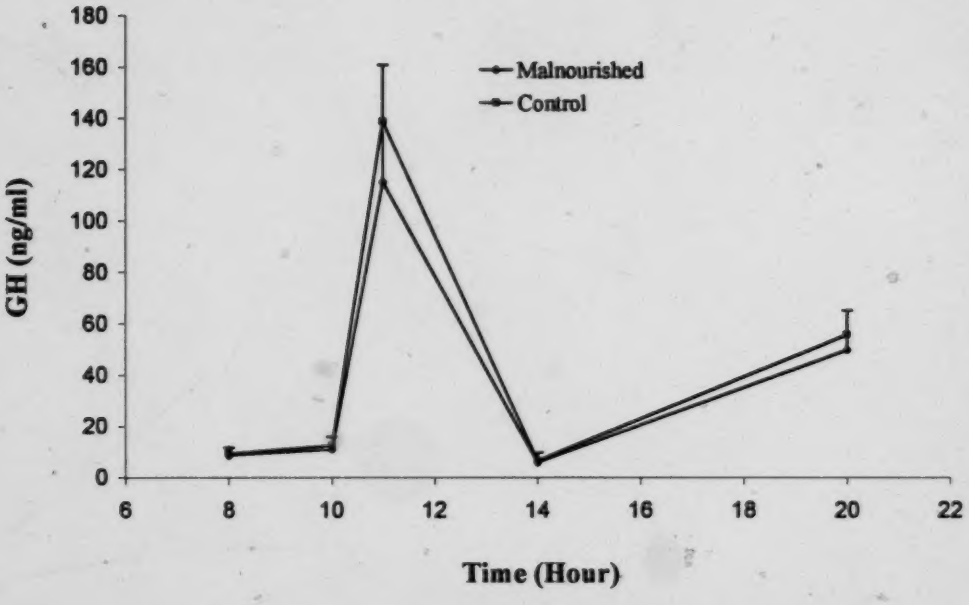

Figure 7.2 Circadian rhythmicity of rGH in adult control and malnourished rats after two months of food restriction (n=6/group). Values are expressed as mean ± SD

CHAPTER 8

SUMMARY AND FUTURE STUDY

A series of *in vitro* and *in vivo* studies was conducted in rats that were protein-calorie malnourished and in malnourished rats treated with parenteral nutrition. The principal objectives were to evaluate the impact of PCM and PN on CYP activities, and therefore on the metabolic elimination of drugs. Testosterone was used as an *in vitro* and nifedipine as an *in vivo* probe. The hypothesis that a change in the rGH pattern during malnutrition was responsible for the change in the rate of drug metabolism was examined.

Before conducting the principal experiments, the animal model was created for the malnutrition study. In the literature, serum albumin was used as a criterion for malnutrition study. The results from this study show that albumin is not a good indicator of malnutrition since it remains unchanged during PCM. Parameters such as microsomal protein, total P450, CYP enzyme capacity and affinity, on the other hand, were changed significantly. Body weight has been found to be a good indicator of malnutrition; rats had an average body weight of 60% of the control, indicating PCM.

A rapid, simple and sensitive reverse-phase high-performance liquid chromatographic (HPLC) assay was developed to quantitate testosterone and its metabolites produced by rat liver microsomes. The technique includes a simple gradient system, which provides a higher resolution of testosterone and its metabolites. Using a LiChrospher 60 RP-select B C_8 column and a simple gradient mobile phase system, all

peaks were well separated and a flat baseline was maintained until all the peaks of interest had been eluted. The procedure not only improved the resolution and quantification of the major testosterone hydroxylated metabolites formed by rat hepatic microsomal systems but also significantly shortened in retention times. The use of this method saves a significant amount of time in routine sample analysis (24 vs. 39 min per run). The developed method can be used to determine CYP activities in rat hepatic microsomes and has been used to monitor alterations in testosterone metabolism in protein-calorie malnourished and parental nutrition-treated rats. This assay may also be used for evaluating hepatic metabolism of new drug candidates and their interactions with other drugs.

It was hypothesized that malnutrition impairs drug metabolism in malnourished pediatric rats. Results from this study showed that PCM decreased levels of microsomal protein and total P450 in pediatric rats. Microsomes of PCM showed impaired 16α and 2α metabolic hydroxylation of testosterone (CYP2C11), testosterone 6β-hydroxylation (CYP3A), and testosterone 7α-hydroxylation (CYP2A1). The V_{max} values were significantly reduced and K_m values were significantly increased, suggesting the capacity and affinity of these reactions were compromised. Testosterone 16β-hydroxylation (CYP2B1) did not show any significant change, either in capacity or affinity in the malnourished pediatric rats. Serum albumin is not a good indicator of malnutrition for such drug metabolism studies since it did not change in PCM.

Similar results were observed in PCM adult rats, but the changes were not as dramatic as those in the pediatric study. Other than serum albumin, total serum protein was also monitored during this study. It was observed that neither serum albumin nor total serum protein levels changed significantly and, therefore, total serum protein is a poor indicator of malnutrition.

PN is used to restore the nutritional status of malnourished subjects. It was postulated that it might also restore a subject's drug metabolism ability. It was observed from the *in vitro* study that PN diminished microsomal protein, total P450 levels, and CYP2C11 and CYP3A activities in healthy animals. Although PN restored the body weight and liver weight of malnourished rats, it further decreased the microsomal protein and total P450 level in malnourished adult rats. PN further impaired the capacity (V_{max}) and affinity (K_m) of the 16α, 2α (CYP2C11) and 6β (CYP3A) testosterone hydroxylation pathways. Based on the results observed in this study, it is clear that although PN can rehabilitate the nutritional status of PCM rats, their drug metabolizing ability is even worse than that of the malnourished animals.

The results of the *in vitro* studies are interesting, we wanted to find out what the story is *in vivo* under malnutrition and PN. Nifedipine pharmacokinetics showed that total body clearance was diminished in PCM rats, the AUC and $t_{1/2}$ of the malnourished rats were much higher than those of the control. V_d was also significantly lower compared to control values.

Observations from parenteral nutrition on nifedipine pharmacokinetics in PCM adult rats showed that parenteral nutrition impaired nifedipine elimination in healthy rats. Parenteral nutrition further impaired nifedipine total body clearance in PCM rats. The values of AUC and $t_{1/2}$ were further increased in PN-treated PCM rats, but volume of distribution did not change significantly in PN rats. It was found that the impairment of drug metabolism involving the CYP2C11 pathway in PN subjects is more pronounced in PCM rats since more than 80% of nifedipine is metabolized by CYP2C11 in male rats and in this study, parenteral nutrition further impaired nifedipine total body clearance in PCM rats.

The rate of metabolism of many drugs is an important and critical factor affecting the intensity and duration of drug action. Diet and nutritional status are two environmental variables that account for a substantial proportion of variation in drug metabolism. Results observed from rat are not necessary all applicable to human since animals and human have different metabolic pathways, such as rat CYP2C11 is not a equivalent of human CYP2C11, instead, it shares most of substrates with human CYP3A4 for metabolism. But, data from this study suggest that for malnourished subjects, drug metabolism was impaired. For malnourished patient, drug dosage adjustments may be required and clinical monitoring of drug administration may be necessary.

Based on the results observed in this study, it is clear that although PN can rehabilitate the nutritional status of PCM rats, an animal's drug metabolizing ability is worse than that of a malnourished animal. If this observation can be extended to humans,

the use of drugs in patients receiving PN who are malnourished should be done with caution, especially when the drugs used are eliminated by the action of the major human CYP isoenzyme, CYP3A4. CYP2C11 in rat and CYP3A4 in human share most substrates for metabolism and CYP2C11 was severely impaired in the nutritional studies reported here.

It was hypothesized that the mechanism involved in the alteration of drug metabolism in PCM rats was due to a change in rGH levels. The investigation of rGH concentrations in PCM rats showed that rGH secretion did not change significantly in SD male rats after two months of food restriction. The quantity and the secretion pattern were not altered in PCM rats compared to the healthy ones. The change of GH is stress-related and has a relationship with serum albumin. It is clear from this study that the impairment of drug metabolism in PCM rats was not caused by an alteration of rGH.

As malnutrition impairs immune system, there is a strong association between malnutrition and inflammation [1]. It was reported that malnourished patients had higher C-reactive protein levels, elevated intima-media area and higher prevalence of carotid plaques compared with well-nourished patients, indicating malnutrition caused inflammation [1]. Inflammation, independent of ideology, causes an elevation of cytokines and pro-inflammatory cytokines and interferons, which differentially regulate P450s [3]. The expression of most P450s in the liver is suppressed, some are induced such as CYP4A during inflammation [1,2]. Mitchell et al. [2] documented that CYP4A mRNAs were induced in the livers and kidneys of rats during food restriction, unlike other hepatic P450

gene products (CYP2C11, CYP3A, CYP2A), which are impaired as observed in the study reported here. The changes in cytokines in malnourished subjects could cause alterations in P450 gene transcription, protein turnover, and RNA production, as cytokines regulate P450 gene transcription, modulate RNA and protein turnover [3]. This couold be one of the mechanisms which caused the alteration in PCM rats. Further investigations are required.

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